

**UTILITY
PATENT APPLICATION
TRANSMITTAL**

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.

3335-075-55 CONT

First Inventor or Application Identifier

JOHN C. REED

Title

REGULATION OF BCL-2 GENE EXPRESSION

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

Fee Transmittal Form (e.g. PTO/SB/17)
(Submit an original and a duplicate for fee processing)

ACCOMPANYING APPLICATION PARTS

6. ☒ Assignment Papers (the prior application is assigned to University of Pennsylvania Reel No: 6835, Frame Nos: 295-297) (copy attached)
7. ☐ 37 C.F.R. §3.73(b) Statement (when there is an assignee) ☐ Power of Attorney
8. ☐ English Translation Document (if applicable)
9. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations
10. ☒ Preliminary Amendment
11. ☒ White Advance Serial No. Postcard
12. ☐ Small Entity Statement(s) ☒ Statement filed in prior application. Status still proper and desired. (copy attached)
13. ☐ Certified Copy of Priority Document(s) (if foreign priority is claimed)
14. ☒ Other: Request For Priority

2. ☒ Specification

Total Pages **80**

3. ☒ Drawing(s) (35 U.S.C. 113) Total Sheets

16

4. ☒ Oath or Declaration

Total Pages

a. ☐ Newly executed (original or copy)

b. ☒ Copy from a prior application (37 C.F.R. §1.63(d))
(for continuation/divisional with box 15 completed)

i. ☐ DELETION OF INVENTOR(S)

Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. §1.63(d)(2) and 1.33(b).

☒ Incorporation By Reference (usable if box 4B is checked)
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4B, is considered to be part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

15. If a CONTINUING APPLICATION, check appropriate box, and supply the requisite information below:

☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application no.: 09/080,285, allowed

Prior application information: Examiner: MCGARRY, S.

Group Art Unit: 1635

16. Amend the specification by inserting before the first line the sentence:

☒ This application is a ☒ Continuation ☐ Division ☐ Continuation-in-part (CIP)

of application Serial No. 09/080,285 Filed on MAY 18, 1998, allowed

Which is a continuation of application Serial No. 08/465,485, filed on June 5, 1995, patented, which is a continuation of application Serial No. 08/124,256, filed September 20, 1993, abandoned, which is a continuation-in-part of application Serial No. 07/840,716, filed February 21, 1992, abandoned, which is a continuation-in-part of application Serial No. 07/288,692, filed December 22, 1988, abandoned.

☐ This application claims priority of provisional application Serial No.

Filed

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Applicant or Patent John C. ReedSerial or Patent No.: 08/124,256Filed or Issued: September 20, 1993For: REGULATION OF bcl-2 GENE EXPRESSION

COPY

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION University of PennsylvaniaADDRESS OF ORGANIZATION 3700 Market Street, Suite 200, Philadelphia, PA 19104

TYPE OF ORGANIZATION

- ☒ University or other institution of higher education
☐ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))
☐ Nonprofit scientific or educational under statute of state
 (Name of State _____)
 (Citation of Statute _____)
☐ Would qualify as tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)) if located in the United States of America
☐ Would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
 (Name of State _____)
 (Citation of Statute _____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled REGULATION OF bcl-2 GENE EXPRESSION

by inventor: **JOHN C. REED**

described in

- [] the specification filed herewith.
 [xx] application serial no. 08/124,256, filed 9/20/93.
 [] patent no. _____, issued _____.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below: and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME _____

ADDRESS _____

[] Individual [] Small Business Concern [] Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

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33. The anticodon oligomer of Claim 32 wherein said anticodon oligomer is a phosphorothioate derivative.

34. The anticodon oligomer of Claim 32, wherein said anticodon oligomer is from about 10 to about 100 nucleotides long.

35. The anticodon oligomer of Claim 34, wherein said anticodon oligomer is from about 15 to about 24 nucleotides in length.

36. The anticodon oligomer of Claim 32, which is complementary to and which binds to a translation-initiation, splicing, transport or degradation site in mRNA or pre-mRNA expressed from the human bcl-2 gene.

37. The anticodon oligomer of Claim 32, selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 14 and SEQ ID NO: 17.

38. The anticodon oligomer of Claim 37, selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5.

39. A composition for inhibiting growth of tumor cells expressing the human bcl-2 gene, comprising:

- (e) the anticodon oligomer of Claim 32, and
- (f) a pharmaceutically acceptable carrier.

40. The composition of Claim 39 wherein said anticodon oligomer is a phosphorothioate derivative.

41. The composition of Claim 39, wherein said anticodon oligomer is from about 10 to about 100 nucleotides in length.

42. The composition of Claim 41, wherein said anticodon oligomer is from about 15 to about 24 nucleotides in length.

43. The composition of Claim 39, wherein said anticodon oligomer is complementary to and binds to a translation, splicing, transport or degradation site in mRNA or pre-mRNA or pre-mRNA expressed from the human bcl-2 gene.

44. The composition of Claim 39, wherein said anticodon oligomer is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 14 and SEQ ID NO: 17.

45. A vector for transfecting human tumor cells wherein said vector expresses the anticodon oligomer of Claim 32.

46. The vector of Claim 45, wherein said anticodon oligomer is from about 10 to about 100 nucleotides in length.

47. The vector of Claim 46, wherein said anticodon oligomer is from about 15 to 24 nucleotides in length.

48. The vector of Claim 45, wherein said anticodon oligomer sequence is complementary to and binds to a translation-initiation, splicing, transport or degradation site in mRNA or pre-mRNA expressed from the human bcl-2 gene.

49. The vector of Claim 45, wherein said anticodon oligomer sequence is selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 14 and SEQ ID NO: 17.

50. The vector of Claim 49, selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5.

51. A kit comprising the composition of Claim 39 and a chemotherapeutic agent.

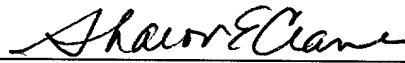
52. A kit comprising the vector of Claim 45 and a chemotherapeutic agent.--

REMARKS

Claims 32-52 are now pending in this application. Early and favorable consideration is respectfully requested.

Respectfully submitted,

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REGULATION OF bcl-2 GENE EXPRESSIONReference to Government Grants

The research in this patent application was supported in part by National Institutes of Health grant CA 26380. The United States government has certain rights in the invention.

Field of the Invention

The present invention relates to the field of treatments for cancer and more particularly to the field of anticode oligomer treatments for cancer.

Related Application Data

This application is a continuation-in-part of Serial No. 07/840,716 filed February 21, 1992, which was a continuation in part of Serial No. 07,288,692 filed December 22, 1988, which has been abandoned.

Background of the Invention

Current approaches to cancer treatment suffer from a lack of specificity. The majority of drugs that have been developed are natural products or derivatives that either block enzyme pathways or randomly interact with DNA. Due to low therapeutic indices, most cancer treatment drugs are accompanied by serious dose-limiting toxicities. The administration of drugs to treat cancer kills not only cancer cells but also normal non-cancerous cells. Because of these deleterious effects, treatments that are more specific for cancerous cells are needed.

It has been found that a class of genes, the oncogenes, plays a large role in the transformation and maintenance of the cancerous state and that turning off these genes, or otherwise inhibiting their effects, can return a cell to a normal phenotype. The role of oncogenes in the etiology of many human cancers has been

reviewed in Bishop, "Cellular Oncogenes and
Retroviruses," *Science*, 235:305-311 (1987). In many
types of human tumors, including lymphomas and leukemias,
the human bcl-2 gene is overexpressed, and may be
5 associated with tumorigenicity (Tsujimoto et al.
Involvement of the bcl-2 gene in human follicular
lymphoma, *Science* 228:1440-1443 (1985)).

Antisense oligodeoxynucleotides are one example
10 of a specific therapeutic tool with the potential for
ablating oncogene function. These short (usually about
30 bases) single-stranded synthetic DNAs have a
complementary base sequence to the target mRNA and form a
hybrid duplex by hydrogen bonded base pairing. This
15 hybridization can be expected to prevent expression of
the target mRNA code into its protein product and thus
preclude subsequent effects of the protein product.
Because the mRNA sequence expressed by the gene is termed
the sense sequence, the complementary sequence is termed
20 the antisense sequence. Under some circumstances,
inhibition of mRNA would be more efficient than
inhibition of an enzyme's active site, since one mRNA
molecule gives rise to multiple protein copies.

Synthetic oligodeoxynucleotides complementary
25 to (antisense) mRNA of the c-myc oncogene have been used
to specifically inhibit production of c-myc protein, thus
arresting the growth of human leukemic cells in vitro,
Holt et al., *Mol. Cell Biol.* 8:963-973 (1988), and
Wickstrom et al., *Proc. Natl. Acad. Sci. USA*,
30 85:1028-1-32 (1988). Oligodeoxynucleotides have also
been employed as specific inhibitors of retroviruses,
including the human immunodeficiency virus (HIV-I),
Zamecnik and Stephenson, *Proc. Natl. Acad. Sci. USA*,
75:280-284 (1978) and Zamecnik et al., *Proc. Natl. Acad.*
35 *Sci. USA*, 83:4143-4146 (1986).

Summary of the Invention

5 The invention provides anticode oligomers and methods for inhibiting growth of cancer cells. The growth of lymphoma or leukemia cells, which are types of lymphocytes, are inhibited by the anticode oligomers and methods of the invention. An anticode oligomer complementary to at least an effective portion of the mRNA sense strand to the human *bcl-2* gene is provided and cells are then contacted with the anticode oligomer in a concentration sufficient to inhibit growth of the cells. 10 The methods of the invention are suitable for inhibiting growth of lymphoma/leukemia cells that express the human *bcl-2* gene and have a t (14; 18) chromosomal translocation as well as those that express the *bcl-2* gene but do not have a t (14; 18) chromosomal translocation. 15

20 In accordance with preferred embodiments, the anticode oligomer is substantially complementary to a strategic site in the pre-mRNA sense strand or substantially complementary to the mRNA. A preferred strategic site is the translation-initiation site of the pre-mRNA coding strand. Alternative strategic sites include coding sites for splicing, transport or degradation. The subject anticode oligomer either in its "native," unmodified form -- oligonucleotide -- or as a derivative, is brought into contact with the target lymphoma or leukemia cells. For *in vivo* therapeutic use, a derivative of the "native" oligonucleotide, such as the phosphorothioate form is preferable since it is believed 25 that these forms are more resistant to degradation, notwithstanding the fact that response times to some analogues, such as the phosphorothioate analogs, has been found to be somewhat slower than to the "native" form of the oligonucleotide. 30

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5 A preferred anticode oligomer, denominated
herein the TI-AS (translation initiation anticode
oligomer) is an oligodeoxynucleotide which straddles the
translation-initiation site of the mRNA coding strand of
the human *bcl-2* gene and is complementary to this region.
More preferably, this nucleotide comprises a TAC portion
which is complementary to the ATG initiation sequence of
the coding strand for the *bcl-2* gene, and preferably
10 further comprises flanking portions of two to about one
hundred bases, more preferably from about five to about
twenty bases, which are complementary to portions of the
bcl-2 gene coding strand flanking said initiation
sequence. The TI-AS nucleotide has been found effective
at inhibiting the growth of the target cells both in the
15 presence and absence of serum.

20 Alternatively, the anticode oligomer comprises
an antisense nucleotide complementary to at least an
effective portion of the splice donor site of the pre-
mRNA coding strand for the human *bcl-2* gene. More
particularly, this nucleotide comprises a CA portion
which is complementary to the GT splice donor of the
bcl-2, and again comprises flanking portions of two to
about one hundred bases, preferably from about five to
about twenty bases, which are complementary to portions
25 of the *bcl-2* gene coding strand flanking said splice
donor.

30 In yet another embodiment, the anticode
oligomer is complementary to at least an effective
portion of the splice acceptor region of the pre-mRNA
coding strand for the human *bcl-2* gene. This oligomer
comprises at least a TC portion which is complementary to
the AG splice acceptor of the *bcl-2* gene, and again
comprises flanking portions of two to about one hundred,
preferably from about five to about twenty bases which
35 are complementary to portions of the *bcl-2* gene coding

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strand flanking said acceptor. The subject oligomer may also be selected to overlap the coding site for the 26 kDa protein, *bcl-2*-alpha or for the 22 kDa protein, *bcl-2*-beta, protein products of the *bcl-2* gene.

5 Preferably the oligomer is selected to minimize homology with anticode oligomers for pre-mRNA or mRNA coding strands for other gene sequences.

Accordingly, a primary object of the present invention is the provision of novel anticode oligomers, which are useful in inhibiting the growth of cancer cells. The present invention also includes compositions for inhibiting the growth of tumor cells, which compositions comprise the anticode oligomer of the present invention together with a pharmaceutically acceptable carrier.

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A further object of the present invention is the provision of methods for inhibiting the growth of cancer cells using said anticode oligomers. As a feature of the present invention, it was discovered that average reductions of 30-40% in the relative levels of *bcl-2* protein markedly enhanced the sensitivity of lymphoma cells, in particular, t(14;18)-containing lymphoma cell lines to cancer chemotherapeutic agents, including conventional anticancer drugs. Such reductions were achieved by introducing into tumor cells an anticode oligomer which binds to either pre-mRNA or mRNA expressed from the *bcl-2* gene. Two methods were used in the present invention to introduce said anticode oligomers to tumor cells. One method involved contacting the tumor cells with a composition comprising the anticode oligomers. Another method involved transfecting the tumor cells with a vector encoding an antisense oligonucleotide. Introducing an anticode oligomer to tumor cells achieved a reduction of *bcl-2* expression and

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25
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increases the chemosensitivity of neoplastic cells to cancer chemotherapeutic agents or anticancer drugs.

Accordingly, the present invention achieved a method of killing tumor cells by introducing to tumor cells anticode oligomers which reduce *bcl-2* gene expression or impair Bcl-2 protein function before contacting the cells with cancer chemotherapeutic agents. The cancer chemotherapeutic agents reduced the numbers of viable malignant cells, and the portion of tumor cells killed was greater than the portion which would have been killed by the same amount of drug in the absence of introducing the anticode oligomer oligodeoxynucleotide to the cells.

These and other objects of the present invention will become apparent from the following detailed description.

Brief Description of the Drawings

Figure 1 shows graphs of the effects of varying concentrations of antisense oligodeoxynucleotides on inhibition of cell proliferation.

Figure 2 shows graphs of the concentration dependence of inhibition of cell proliferation by antisense normal and phosphorothioate oligodeoxynucleotides. Oligodeoxynucleotide additions to cultures included TI-AS phosphorothioate (○ and ●; two separate experiments), TI-S phosphorothioate (▲), TI-AS normal (□), and TI-S normal (Δ).

Figure 3 shows the results of gel electrophoresis of six antisense oligonucleotides targeted against the translation initiation site of *bcl-2* mRNA.

Figure 4 shows the degree of DNA fragmentation resulting from oligonucleotide treatment of RS11846 cells. Figure 4(a) shows the effect of oligonucleotides targeted against the translation initiation site.

5 Figure 4(b) shows the effect of oligonucleotides directed against the 5'-cap region of *bcl-2* mRNA.

Figure 5 is a graph showing the concentration--dependence of inhibition by an antisense oligonucleotide targeted against the translation initiation site of *bcl-2* mRNA.

10

Figures 6 (a) and (b) are graphs showing the results of immunofluorescence analysis of *bcl-2* protein levels in oligonucleotide-treated cells.

Figures 7 (a)-(d) are FACS profiles for 697 cells before and after treatment with *bcl-2* antisense oligonucleotides.

15

Figure 8 (a) - (c) show *bcl-2* antisense oligodeoxynucleotides producing sequence-specific reductions in *bcl-2* mRNA and *bcl-2* protein and producing increased sensitivity of SU-DHL-4 cells to cancer chemotherapeutic drugs.

20

Figure 9 demonstrates the differential effects of *bcl-2* antisense oligomers on chemosensitivity of 32D-*bcl-2* and 32D-BHRF-1 cells.

Figure 10 (a-b) shows reduction of chemoresistance of RS11846 cells from inducible *bcl-2* antisense expression from an expression plasmid.

25

Figure 11 shows methylphosphonate/phosphodiester *bcl-2* antisense oligomers inducing death of DOHH2 lymphoma cells.

30

Figure 12 shows methylphosphonate (MP)/Phosphodiester (PO) chimeric oligomers inhibiting growth of MCF-7 human breast cancer cells.

5 Figure 13 shows optimization of antisense bcl-2 oligomer sequences.

Detailed Description of the Invention

According to the invention, anticode oligomers are provided for inhibiting cancer cell growth, for increasing the sensitivity of cancer cells to cancer
10 chemotherapeutic agents, or for inducing cancer cell death alone or in combination with any one or more cancer chemotherapeutic agents.

Definitions

As used herein, the term "anticode oligomers"
15 means anticode oligonucleotides and analogs thereof and refers to a range of chemical species that recognize polynucleotide target sequences through hydrogen bonding interactions with the nucleotide bases of the target
20 sequences. The target sequences may be single- or double-stranded RNA or single- or double-stranded DNA.

The anticode oligonucleotides and analogs thereof may be RNA or DNA, or analogs of RNA or DNA, commonly referred to as antisense oligomers or antisense oligonucleotides. Such RNA or DNA analogs comprise but
25 are not limited to 2-'O-alkyl sugar modifications, methylphosphonate, phosphorothiate, phosphordithioate, formacetal, 3'-thioformacetal, sulfone, sulfamate, and nitroxide backbone modifications, and analogs wherein the base moieties have been modified. In addition, analogs
30 of oligomers may be polymers in which the sugar moiety has been modified or replaced by another suitable moiety, resulting in polymers which include, but are not limited to, morpholino analogs and peptide nucleic acid (PNA)

analogs (Egholm, et al. Peptide Nucleic Acids (PNA) -
Oligonucleotide Analogues with an Achiral Peptide
Backbone, (1992)).

Anticodons may also be mixtures of any of
5 the oligonucleotide analog types together or in
combination with native DNA or RNA. At the same time,
the oligonucleotides and analogs thereof may be used
alone or in combination with one or more additional
oligonucleotides or analogs thereof. The
10 oligonucleotides may be from about 10 to about 1,000
nucleotides long. Although oligonucleotides of 10 to 100
nucleotides are useful in the invention, preferred
oligonucleotides range from about 15 to about 24 bases in
length.

Anticodon oligonucleotides and analogs thereof
15 also comprise conjugates of the oligonucleotides and
analogs thereof. (John Goodchild, Conjugates of
Oligonucleotides and Modified Oligonucleotides: A Review
of Their Synthesis and Properties, Bioconjugate
20 Chemistry, Volume 1 No. 3, May/June (1990)). Such
conjugates having properties to improve the uptake,
pharmacokinetics, and nuclease resistance of the
oligonucleotide, or the ability to enhance cross-linking
or cleavage of the target sequence by the
25 oligonucleotide.

As used herein, the term "cell proliferation"
refers to cell division rate/cell cycle. The term
"growth," as used herein, encompasses both increased cell
numbers due to faster cell division and due to slower
30 rates of cell death.

As used herein, bcl-2 gene expression refers to
bcl-2 protein production from the human bcl-2 gene; e.g.

reduced *bcl-2* gene expression means reduced levels of *bcl-2* protein.

As used herein, "strategic sites" are defined as any site which when bound by the claimed anticode molecules or analogs thereof results in inhibiting expression of the *bcl-2* gene.

As used herein, the term "sequence portion" is a portion of the nucleotide sequence of an RNA oligonucleotide. In appropriate contexts, "sequence portion" may refer to a portion of the nucleotide sequence of a DNA segment or DNA oligonucleotide.

Uncontrolled cell proliferation is a marker for a cancerous or abnormal cell type. Normal, non-cancerous cells divide regularly, at a frequency characteristic for the particular type of cell. When a cell has been transformed into a cancerous state, the cell divides and proliferates uncontrollably. Inhibition of proliferation modulates the uncontrolled division of the cell. Containment of cell division often correlates with a return to a non-cancerous state.

A human gene termed *bcl-2* (B cell lymphoma/leukemia-2) is implicated in the etiology of some common lymphoid tumors, Croce et al., "Molecular Basis Of Human B and T Cell Neoplasia," in: *Advance in Viral Oncology*, 7:35-51, G. Klein (ed.), New York: Raven Press, 1987. High levels of expression of the human *bcl-2* gene have been found in all lymphomas with t (14; 18) chromosomal translocations including most follicular B cell lymphomas and many large cell non-Hodgkin's lymphomas. High levels of expression of the *bcl-2* gene have also been found in certain leukemias that do not have a t(14; 18) chromosomal translocation, including most cases of chronic lymphocytic leukemia acute, many

lymphocytic leukemias of the pre-B cell type,
neuroblastomas, nasopharyngeal carcinomas, and many
adenocarcinomas of the prostate, breast, and colon.
(Reed et al., Differential expression of bcl-2
5 protooncogene in neuroblastoma and other human tumor cell
lines of neural origin. Cancer Res. 51:6529 (1991); Yunis
et al. Bcl-2 and other genomic alterations in the
prognosis of large-cell lymphomas. New England J. Med.
320:1047; Campos et al. High expression of bcl-2 protein
10 in acute myeloid leukemia is associated with poor
response to chemotherapy. Blood 81:3091-3096 (1993);
McDonnell et al. Expression of the protooncogene bcl-2
and its association with emergence of androgen-
independent prostate cancer. Cancer Res. 52:6940-6944
15 (1992); Lu Q-L, et al. Bcl-2 protooncogene expression
in Epstein Barr Virus-Associated Nasopharyngeal
Carcinoma, Int. J Cancer 53:29-35 (1993); Bonner et al.
bcl-2 protooncogene and the gastrointestinal mucosal
epithelial tumor progression model as related to proposed
20 morphologic and molecular sequences, Lab Invest. 68:43A
(1993)).

While not limited to the following explanation,
the present invention exploits cellular mechanisms
concerned with normal cell death. Because most types of
25 cells have a finite life span and are programmed to die,
uncontrollable cell accumulation can also result because
of a defect in normal cell death mechanisms rather than
through an increased rate of cell division. The bcl-2
gene contributes to the pathogenesis of cancer primarily
30 by prolonging cell survival rather than accelerating cell
division.

Antisense oligomers suitable for use in the
invention include nucleotide oligomers which are two to
two hundred nucleotide bases long; more preferably ten to
35 forty bases long; most preferably twenty bases long. The

oligonucleotides are preferably selected from those
oligonucleotides complementary to strategic sites along
the pre-mRNA of *bcl-2*, such as the translation initiation
site, donor and splicing sites, or sites for
5 transportation or degradation.

Blocking translation at such strategic sites
prevents formation of a functional *bcl-2* gene product.
It should be appreciated, however, that any combination
or subcombination of anticode oligomers, including
10 oligonucleotides complementary or substantially
complementary to the *bcl-2* pre-mRNA or mRNA that inhibit
cell proliferation is suitable for use in the invention.
For example, oligodeoxynucleotides complementary to
sequence portions of contiguous or non-contiguous
15 stretches of the *bcl-2* RNA may inhibit cell proliferation
and would thus be suitable for use in the invention.

It should also be appreciated that anticode
oligomers suitable for use in the invention may also
include oligonucleotides flanking those complementary or
20 substantially complementary to such sequence portions as
the strategic or other sites along the *bcl-2* mRNA. The
flanking sequence portions are preferably from two to
about one hundred bases, more preferably from about five
to about twenty bases in length. It is also preferable
25 that the anticode oligomers be complementary to a
sequence portion of the pre-mRNA or mRNA that is not
commonly found in pre-mRNA or mRNA of other genes to
minimize homology of anticode oligomers for pre-mRNA or
mRNA coding strands from other genes.

30 Preferred antisense, or complementary,
oligodeoxynucleotides are listed in Table 1.

TABLE I

bcl-2 Oligodeoxynucleotides

	translation initiation	
	antisense (TI-AS)	3'...CCCTTCCTACCGCGTGCGAC...5'
5	<i>bcl-2</i>	5'...CTTTTCCTCTGGGAAGGATGGCGCACGCTGGGAGA...3'
	splice donor	
	antisense (SD-AS)	31...CCTCCGACCCATCCACGTAG...5'
	<i>bcl-2</i>	5'...ACGGGGTAC...GGAGGCTGGGTAGGTGCATCTGGT...3'
	splice acceptor	
10	antisense (SA-AS)	3'...GTTGACGTCCTACGGAAACA...5'
	<i>bcl-2</i>	5'...CCCCCAACTGCAGGATGCCTTTGTGGAAGTGTACGG...3'

It will be appreciated by those skilled in the art to which this invention pertains, that anticode oligomers having a greater or lesser number of substituent nucleotides, or that extend further along the *bcl-2* mRNA in either the 3' or 5' direction than the preferred embodiments, but which also inhibit cell proliferation are also within the scope of the invention.

It is preferable to use chemically modified derivatives or analogs of anticode oligomers in the performance of the invention rather than "native" or unmodified oligodeoxynucleotides. "Native" oligodeoxynucleotides can be conveniently synthesized with a DNA synthesizer using standard phosphoramidite chemistry. Suitable derivatives, and methods for preparing the derivatives, include phosphorothioate, Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988); methylphosphonate, Blake et al., *Biochemistry* 24:6132-6138 (1985) and aldehydeoxynucleotides, Morvan et al., *Nucl. Acids Res.* 14:5019-5032 (1986), 2'-O-methyl-ribonucleosides (Monia et al. Evaluation of 2'-modified oligonucleotides containing 2' deoxy gaps as antisense inhibitors of gene expression. *J. Biol. Chem.* 268:14514-14522 (1993)), and covalently-linked derivatives such as

CCCTTCCTACCGCGTGCGAC

acridine, Asseline et al., *Proc. Natl Acad. Sci. USA* 81:3297-3201 (1984); alkylated (e.g., N-2-chlorocethylamine), Knorre et al., *Biochemie* 67:783-789 (1985) and Vlassov et al., *Nucl. Acids Res.* 14:4065-4076 (1986); phenazine, Knorre et al., *supra*, and Vlassov et al., *supra*; 5-methyl-N⁴-N⁴-ethanocytosine, Webb et al., *Nucl. Acids Res.* 14:7661-7674 (1986); Fe-ethylenediamine tetraacetic acid (EDTA) and analogues, Boutorin et al., *FEBS Letter's* 172:43-46 (1984); 5-glycylamido-1, 10-o-phenanthroline, Chi-Hong et al., *Proc. Natl. Acad. Sci. USA* 83:7147-7151 (1986); and diethylenetriamine-pentaacetic acid (DTPA) derivatives, Chu et al., *Proc. Natl. Acad. Sci.* 82:963-967 (1985). All of the above publications are hereby specifically incorporated by reference as if fully set forth herein.

The anticode oligomer of the present invention can also be combined with a pharmaceutically acceptable carrier for administration to a subject or for ex-vivo administration. Examples of suitable pharmaceutical carriers are a variety of cationic lipids, including, but not limited to N-(1-2,3-dioleoyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)]. Liposomes are also suitable carriers for the anticode oligomers of the invention.

The anticode oligomers may be administered to patients by any effective route, including intravenous, intramuscular, intrathecal, intranasal, intraperitoneal, subcutaneous injection, in situ injection and oral administration. Oral administration requires enteric coatings to protect the claimed anticode molecules and analogs thereof from degradation along the gastrointestinal tract. The anticode oligomers may be mixed with an amount of a physiologically acceptable carrier or diluent, such as a saline solution or other

suitable liquid. The anticode oligomers may also be combined with liposomes or other carrier means to protect the anticode molecules or analogs thereof from degradation until they reach their targets and/or
5 facilitate movement of the anticode molecules or analogs thereof across tissue barriers.

The anticode oligomers may also be useful for ex vivo bone marrow purging. Normally, the amounts of conventional cancer chemotherapeutic agents or drugs and
10 irradiation that a patient can receive are limited by toxicity to the marrow, i.e., anemia (fatigue, heart failure), thrombocytopenia (bleeding), neutropenia (infection). Thus, in order to deliver sufficient concentrations of drugs and irradiation to totally
15 eradicate the tumor, the physician would simultaneously destroy the patient's normal bone marrow cells leading to patient demise. Alternatively, large amounts of bone marrow can be surgically extracted from the patient and stored in vitro. while the patient receives aggressive
20 conventional treatment. The patient can then be rescued by reinfusion of their own bone marrow cells, but only if that marrow has been "purged" of residual malignant cells. The claimed anticode oligomers could be used to remove residual malignant cells from the bone marrow.

The anticode oligomers are administered in amounts effective to inhibit cancer or neoplastic cell growth. The actual amount of any particular anticode oligomer administered will depend on factors such as the type of cancer, the toxicity of the anticode oligomer to
30 other cells of the body, its rate of uptake by cancer cells, and the weight and age of the individual to whom the anticode oligomer is administered. Because of inhibitors present in human serum that may interfere with the action of the anticode oligomer an effective amount
35 of the anticode oligomer for each individual may vary.

An effective dosage for the patient can be ascertained by conventional methods such as incrementally increasing the dosage of the anticode oligomer from an amount ineffective to inhibit cell proliferation to an effective amount. It is expected that concentrations presented to cancer cells in the range of about 0.001 micromolar to about 100 micromolar will be effective to inhibit cell proliferation.

The anticode oligomers are administered to the patient for at least a time sufficient to inhibit proliferation of the cancer cells. The anticode oligomers are preferably administered to patients at a frequency sufficient to maintain the level of anticode oligomers at an effective level in or around the cancer cells. To maintain an effective level, it may be necessary to administer the anticode oligomers several times a day, daily or at less frequent intervals. Anticod oligomers are administered until cancer cells can no longer be detected, or have been reduced in number such that further treatment provides no significant reduction in number, or the cells have been reduced to a number manageable by surgery or other treatments. The length of time that the anticod oligomers are administered will depend on factors such as the rate of uptake of the particular oligodeoxynucleotide by cancer cells and time needed for the cells to respond to the oligodeoxynucleotide. *In vitro*, maximal inhibition of neoplastic cell growth by "native," unmodified anticod oligomers occurred two days after initiation of cultures,, whereas phosphorothioate oligodeoxynucleotides required 4 to 7 days to achieve maximal inhibition. *In vivo*, the time necessary for maximal inhibition of cell proliferation may be shorter or longer.

The anticode oligomers of the invention may be administered to patients as a combination of two or more different anticode oligomer oligodeoxynucleotide sequences or as a single type of sequence. For instance,
5 TI-AS and SD-AS could be administered to a patient or TI-AS alone.

It is also believed that the anticode oligomers of the invention may be useful in the treatment of autoimmune diseases. Autoimmune diseases are those
10 diseases in which the body's immune system has malfunctioned in some way. Administration of the anticode oligomers of the invention to a person having an autoimmune disease should inhibit proliferation of bcl-2 overexpressing lymphocytes, which would in turn reduce
15 the symptoms of the autoimmune disease. For use in treating autoimmune diseases, the anticode oligomers would be administered as described herein.

EXAMPLES

General Methods

20 The Examples below use the following protocols:

A. Cells and Cell Cultures. Human leukemic cells lines used for these studies were RS11846 follicular lymphoma cells, 697 pre-B cell acute lymphocytic leukemic cells, and JURAT T cell acute
25 lymphocytic leukemic cells as described in Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986) and Weiss et al., *Proc. Natl. Acad. Sci. USA*, 138:2169-2174 (1987). Human peripheral blood lymphocytes (PBL) were isolated from fresh whole blood as described in Reed et
30 al., *J. Immunol.*, 134:314-319 (1985). All lymphoid cells were cultured at 5×10^5 cells/ml in RPMI medium supplemented with 1 mM glutamine, antibiotics, and either 5-10% (v:v) fetal bovine serum (FBS), 5-10% (v:v) calf serum (CS) (both from Hyclone Laboratories), or 1% (v:v)
35 HLI concentrated supplement (Ventrex Laboratories) for

serum-free cultures. Murine fibroblast cell lines were added at 10^3 cells/cm² in DMEM medium containing glutamine, antibiotics and 5-10% (v:v) FCS. Fibroblast cell lines were NIH 3T3 cells, 3T3-B-alpha-S cells, and 3T3-B-alpha-AS cells. These latter two cell lines are NIH 3T3 cells that express high levels of a human *bcl-2*-alpha cDNA in either the sense or antisense orientation, respectively, by virtue of stable transfection with expression vectors constructs.

B. Measurement of Cellular Growth. Growth of cell lines cultured in the presence or absence of anticodon oligomers was measured by two methods: cell counts using a hemocytometer; and DNA synthesis by assaying [³H]-thymidine incorporation essentially as described in Reed et al., *J. Immunol.*, 134:314-319 (1985). Briefly, cells were cultured in 96-well flat-bottomed microtiter plates (Falcon) at 0.2 ml/well. At appropriate times, cells were resuspended, 25 μ l removed from cultures for cell counting, and this volume replaced with 25 μ l of 20 UCi/ML [³H]-thymidine (specific activity 6.7 Ci/mmol) (New England Nuclear). Microtiter cultures were then returned to 37°C and 95% air: 5% CO₂ atmosphere for 8 hours before lysing cells on glass filters and determining relative levels of [³H]-thymidine incorporation into DNA by scintillation counting. Cell counts were performed in the presence of trypan blue dye to determine the concentration of viable cells in duplicate microcultures.

MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] dye reduction assays were performed by the method of Tada, et al. *J. Immunol Methods* 93, 157 (1986), and confirmed to be within the linear range of the assay under the conditions described here. The number of viable cells per well was extrapolated from standard curves that were included with each assay and

that consisted of serial two-fold dilutions of exponentially growing SU-DHL-4 cells in HL-1 medium, beginning with 10^6 cells/ml (0.2ml/well). Samples were assayed in triplicate and the OD600_{nm} for a media/reagent blank was subtracted from all values prior to calculations.

C. RNA Blot Analysis. Total cellular RNA was isolated by a guanidinium isothiocyanate/phenol procedure as described in Chomczynski et al., *Analyt. Biochem.*, 162:156-139 (1987). The polyadenylated fraction was purified by oligodeoxythymidine-cellulose chromatography as described in Aviv et al., *Proc. Natl. Acad. Sci. USA*, 69:1408-1412 (1972). Approximately 5 μ g aliquots of mRNA were size-fractionated in 0.8% agarose/6% formaldehyde gels and transferred to nylon membranes. Blots were prehybridized, hybridized, and washed exactly as described in Reed et al., *Mol. Cell Biol.*, 5:3361-3366 (1985), using either a 32 P-cDNA for human *bcl-2*, as described in Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986), or a murine *bcl-2* probe, pMBCL5.4 as described in Negrini et al., *Cell*, 49:455-463 (1987). Blots were exposed to Kodak XAR film with intensifying screens at -70°C for 1-10 days. Eluting 32 P-*bcl-2* probes from membranes and rehybridizing with a 32 P probe for mouse beta-2-microglobulin verified nearly equivalent amounts of mRNA for all samples on blots.

EXAMPLE 1

Preparation of Anticodon oligomers

Normal and phosphorothioate

oligodeoxynucleotides were synthesized using an Applied Biosystems 380B DNA synthesizer, and purified by HPLC reverse-phase chromatography (PRP-1 column) as described in Stein et al., *Nucl. Acids Res.*, 16:3209-3221 (1988) which is specifically incorporated as if fully set forth herein. In some cases it was necessary to further purify

oligodeoxynucleotides by C18-Sep-Pak chromatography (Waters Associates, Millipore, Inc.), as described previously in Kern et al. , *J. Clin. Invest.*, 81:237-244 (1988), to eliminate nonspecific cytotoxic activity.

5 Oligodeoxynucleotides eluted in 30% acetonitrile were evaporated to dryness, resuspended at 1-2 mM in sterile Dulbecco's phosphate-buffered saline or Hanks' buffered salt solution (both from Gibco), and stored at -80°C in small aliquots.

10 Table 1 shows the oligodeoxynucleotides synthesized and their relation to the sense-strand of the human *bcl-2* gene. Portions of the sequence of the coding strand of the human *bcl-2* gene are shown, including the translation initiation site (top), splice donor site
15 (middle), splice acceptor region (bottom), and empirically selected sites within the 5' untranslated portion of *bcl-2* pre-mRNA. The ATG initiation codon, GT splice donor, and AG splice acceptor consensus sequences are in boxes.

20 The sequences of the oligodeoxynucleotides synthesized for these investigations are presented, and their relation to human *bcl-2* mRNA is indicated. The TI-AS oligodeoxynucleotide is antisense at the translation initiation site and TI-S is its complementary
25 sense version. SD-AS and SD-S are oligodeoxynucleotides having antisense and sense orientations, respectively, relative to the splice donor region.

The oligodeoxynucleotide TI-AS straddles the predicted translation-initiation site of *bcl-2* mRNAs and
30 is complementary (antisense) to this region. As a control, the sense version of this 20 bp oligodeoxynucleotide, TI-S, was also synthesized.

In an effort, to specifically block splicing of *bcl-2* mRNAs, a 20 bp antisense oligodeoxynucleotide, SD-AS, was synthesized that overlaps the splice donor site in *bcl-2* primary transcripts. In addition, a complementary sense oligodeoxynucleotide, SD-S, was prepared as depicted in Table 1. The human *bcl-2* gene gives rise to several transcripts through alternative splice site selections, see Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986). The preponderance of these transcripts depend upon splicing and encode a 26 kDa protein, *bcl-2*-alpha. One minor transcript, however, does not undergo a splice and consequently encodes a 22 kDa protein *bcl-2*-beta. The SD-AS oligodeoxynucleotide can thus potentially block maturation of most but not all *bcl-2* transcripts.

EXAMPLE 2

Treatment of Serum for In Vitro Investigations of Antisense Normal Oligodeoxynucleotides

Because normal oligodeoxynucleotides are sensitive to degradation by nucleases present in serum, the efficacy of the TI-AS oligodeoxynucleotide in fetal bovine serum (FBS) heated for 30 minutes at 56°C (the usual procedure for inactivating serum complement) was contrasted with the efficacy of TI-AS in FBS heated for 1 hour at 68°C, a temperature sufficient for irreversible inactivation of many nucleases. The RS11846 follicular lymphoma cell line was used. RS11846 cells contain a t(14; 18) chromosomal translocation that deregulates *bcl-2* expression, resulting in the accumulation of high levels of *bcl-2* mRNAs, Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986).

RS11846 follicular lymphoma cells were cultured in medium containing 5% (vol:vol) fetal bovine serum (FBS) that had been heated at 56°C for 0.5 hours or at 68°C for 1 hour. TI-AS normal oligodeoxynucleotide was

added at the initiation of culture, and the density of viable cells determined two days later.

5 The TI-AS normal oligodeoxynucleotide was more effective in 68 C-treated serum at suppressing the growth in culture of these lymphoma cells. In all subsequent experiments, sera heated at 68°C for 1 hour prior to use were used in cultures. This treatment did not impair the growth-supporting capacity of the sera.

EXAMPLE 3

10 Specific Inhibition of Lymphoid Cell Growth by Antisense Normal Oligodeoxynucleotides

Antisense normal oligodeoxynucleotides directed against the translation initiation site (TI-AS) and the splice donor site (SD-AS) of *bcl-2* transcripts were
15 tested for their ability to suppress the proliferation of normal and neoplastic lymphoid cells.

RS11846 follicular lymphoma cells, JUKRAT T cell leukemia cells, and freshly isolated peripheral blood lymphocytes were cultured in medium containing 10%
20 (vol:vol) FBS that had been heated at 68°C for one hour. various concentrations of normal oligodeoxynucleotides were added at the initiation of culture, including: TI-AS, TI-S, SD-AS, and SD-S. Relative DNA synthesis was measured in cultures after 2-3 days by [³H]-thymidine
25 incorporation. Data were calculated as a percentage of control cultures containing volumes of PBS or HBSS equivalent to oligodeoxynucleotide-treated cultures, and represent the mean (\pm standard deviation) of duplicate cultures.

30 Similar data were obtained by measuring cell counts, excluding cold thymidine inhibition as an explanation for the suppression of DNA synthesis observed in cultures treated with antisense oligodeoxynucleotides.

003344 000000

As shown in Figure 1, both the TI-AS and SD-AS oligodeoxynucleotides inhibited the growth of RS11846 cells in a concentration-dependent manner. The SD-AS oligonucleotide was less effective in inhibiting cell growth than the TI-AS oligodeoxynucleotide. In contrast to these antisense oligodeoxynucleotides, sense oligodeoxynucleotides (TI-S and SD-S) were not inhibitory even at concentrations of up to 250 μ G/ml. Moreover, non-sense oligodeoxynucleotides (i.e., those having the same base composition as the antisense oligodeoxynucleotides but with scrambled sequences) also failed to suppress the proliferation of RS11846 cells. The data thus indicate that antisense oligodeoxynucleotides can specifically block the proliferation of these tumor cells. Several other leukemic cell lines that express the *bcl-2* gene were also tested for inhibition of their proliferation by TI-AS and SD-AS oligonucleotides. As with the JURKAT T cell acute lymphocytic leukemic cells, in every case a specific and concentration-dependent decrease in the growth of these human leukemic cells in cultures containing antisense oligodeoxynucleotides was observed.

It has been demonstrated that *bcl-2* expression is transiently induced in normal human peripheral blood lymphocytes (PBL) when these cells are stimulated to proliferate, suggesting that this gene may play a role in the regulation of normal lymphocyte growth, Reed et al., *Science* 236:1295-1297 (1987). The capacity of antisense oligodeoxynucleotides to impair the growth of PBL cultured with a monoclonal antibody, OKT3 (Van den Elsen et al., *Nature* 312:413-418 (1984)), that stimulates their proliferation was therefore tested. PBL were stimulated with 50 μ l of purified OKT3 monoclonal antibody. As shown in Figure 1, the TI-AS oligodeoxynucleotide specifically suppressed the proliferation of PBL in a concentration-dependent manner. These antisense normal

oligodeoxynucleotides thus suppressed the growth in culture of leukemic cells that constitutively express the *bcl-2* gene and of normal lymphocytes where in *bcl-2* expression is inducible.

5

EXAMPLE 4

Time-Course of Inhibition by

Antisense Normal Oligodeoxynucleotides

The kinetics of inhibition by antisense oligodeoxynucleotides was examined in cultures of RS11846
10 follicular lymphoma cells and of 697 pre-B cell acute lymphocytic leukemia cells. Both of these neoplastic B cell lines transcribe and accumulate *bcl-2* mRNAs at high levels, Tsujimoto et al., *Proc. Natl. Acad. Sci. USA*, 83:5214-5218 (1986).

15 RS11846 follicular lymphoma and 697 pre-B cell leukemia cells were cultured in medium containing 10% (vol:vol) 68°C-treated FBS and normal oligodeoxynucleotides. Cells were cultured with 50 µg/ml
20 TI-AS, 100 µg/ml SD-AS, 50 µg/ml TI-S (RS11846 cells) or 100 µg/ml SO-S (697 cells), or PBS as a control. DNA synthesis (kcpm/10⁵ viable cells) and cell densities (10⁵ viable cells/ml) were measured at various times after initiation of cultures.

Antisense normal oligodeoxynucleotides markedly
25 inhibited DNA synthesis measured in cultures of these cells within 24 hours. Diminished cell densities were readily apparent in these cultures within 2 days. Antisense normal oligodeoxynucleotides thus rapidly inhibited the in vitro growth of leukemic cells. The
30 action of antisense oligodeoxynucleotides was specific, since sense oligodeoxynucleotides did not impair proliferation in these cultures. Though cell viabilities often declined during the later days of culture no increase in cell death was seen during the first 1-2 days

of culture with antisense oligodeoxynucleotides, suggesting a non-cytotoxic mechanism.

EXAMPLE 5

Comparison of Different Serum Preparations

5 Inhibition of proliferation of leukemic cells with antisense oligodeoxynucleotides can vary greatly depending on the lot of serum used in cultures.

10 To determine the effects of serum of inhibition of proliferation, relative levels of DNA synthesis were measured in cultures of 697 pre-B cell leukemia cells 2 days after addition of 200 μ M TI-AS normal oligodeoxynucleotide. Cells were cultured in medium supplemented with 1% (vol:vol) HL1-concentrate (serum-free condition), 5% (vol:vol) of two different
15 lots of calf serum (CS1 and CS2), or 5% (vol:vol) of two different lots of fetal bovine serum (FBS1 and FBS2). All sera were heated at 68°C for 1 hour prior to use in cultures.

20 The normal TI-AS oligodeoxynucleotide markedly inhibited DNA synthesis (92%) and cellular proliferation in serum-free cultures (HL1) of 697 cells. This antisense oligodeoxynucleotide was equally effective (94%) in cultures containing 5% (v:v) of one of the lots of fetal bovine serum (FBS2). In contrast, inhibition
25 was significantly reduced in cultures containing other serum preparations (CS1, CS2, FBS1). It has been generally observed that antisense normal oligodeoxynucleotides are less effective in cultures supplemented with calf serum (CS) than in those
30 containing fetal bovine serum (FBS).

EXAMPLE 6

Concentration Dependence of Inhibition by Antisense
Normal Oligodeoxynucleotides in Serum-Free Cultures

697 pre-B cell leukemia cells were cultured in
5 medium with either 1% (vol:vol) HL1-concentrate
(serum-free conditions or 5% (vol:vol) 68°C-treated
FBS2). Relative levels of DNA synthesis and cellular
densities measured after 2 days in cultures containing
various concentrations of normal TI-AS
10 oligodeoxynucleotide.

The TI-AS oligodeoxynucleotide was inhibitory
at lower concentrations when used in serum-free cultures.
At 100 μ M, for instance, no inhibition of cellular
proliferation was seen in FBS2-containing cultures,
15 whereas cell counts were reduced by approximately 75% in
serum-free cultures. At higher concentrations of
antisense oligodeoxynucleotides (200-250 μ M), however,
inhibition of 697 cellular proliferation was comparable
in both types of cultures. The increased efficacy of
20 normal oligodeoxynucleotides in serum-free cultures was
specific, since the sense oligonucleotide (TI-S) was not
inhibitory at the same concentrations.

EXAMPLE 7

Antisense Phosphorothioate Oligodeoxynucleotides:

25 Time Course of Inhibition

To contrast the efficacy of phosphorothioate
oligodeoxynucleotides with that of normal
oligodeoxynucleotides with regard to inhibition of human
leukemic cell growth, phosphorothioate
30 oligodeoxynucleotides were cultured with 697 pre-B cell
leukemia cells and the effects on inhibition were
measured. 697 pre-B cell leukemia cells were cultured in
serum-free medium for various times before measuring DNA
synthesis (kcpm) and cell densities (10^6 cells/ml). Cells
35 were seeded at an initial density of either 0.2×10^5

cells/ml or 0.5×10^5 cells/ml. Culture conditions were 25 μ M TI-AS phosphorathioate, 25 μ M TI-S phosphorothioate, and control cultures treated with HBSS.

To avoid experimental variation due to differences among lots of sera, 697 leukemic cells were cultured in serum-free conditions. When cultured at an initial seeding density of 0.5×10^6 cells/ml, 697 cells achieved maximal DNA synthesis and cellular densities at 4-5 days. Addition of 25 μ M sense phosphorothioate oligodeoxynucleotide (TI-S) at the initiation of these cultures had little effect on 697 cell growth. In replicate cultures containing 25 μ M antisense phosphorothioate (TI-AS), however, some diminution in DNA synthesis was evident within 2 days and was maximal at 4-5 days. Maximal inhibition of 697 cell growth, as determined by cell counts, was seen at 6 days after initiation of cultures.

When 697 cells were initially seeded at 0.2×10^6 cells/ml, the antisense phosphorothioate oligodeoxynucleotide, TI-AS, resulted in only slight inhibition at 2 days, attaining maximal suppression of DNA synthesis in these cultures at day 7. As with normal oligodeoxynucleotides, this inhibition by phosphorothioate oligodeoxynucleotides appeared to be mediated through non-cytotoxic mechanisms, since cellular viabilities did not decline until late in the course of culture. Compared with normal antisense oligodeoxynucleotides, therefore, phosphorothioate oligodeoxynucleotides had a slower onset of action.

EXAMPLE 8

Concentration Dependence of Inhibition by Antisense bcl-2
Phosphorothioate Oligodeoxynucleotides

5 The concentration dependence of inhibition by
phosphorothioate and normal TI-AS oligodeoxynucleotides
in cultures of 697 cells in serum-free medium was
compared as follows.

697 cells were cultured in serum-free medium
for either 3 days (normal oligodeoxynucleotides) or
10 4 days (phosphorothioate oligodeoxynucleotides) prior to
measuring cell densities and levels of DNA synthesis.
Oligodeoxynucleotide additions to cultures included TI-AS
phosphorothioate, TI-S phosphorothioate, TI-AS normal,
and TI-S normal.

15 As shown in Figure 2, TI-AS phosphorothioate
oligodeoxynucleotides markedly inhibited the
proliferation of 697 cells at 25-50 μ M. In contrast,
normal TI-AS oligodeoxynucleotides required
concentrations 5- to 10-fold higher (approximately 250
20 μ M) to cause a comparable suppression of 697 cellular
proliferation. Suppression by the antisense
phosphorothioate oligodeoxynucleotide TI-AS was specific
over this concentration range, since its complementary
sense oligodeoxynucleotide (TI-S) produced little
25 inhibition of 697 cell growth in replicate cultures (see
Figure 2).

EXAMPLE 9

Influence of Serum Preparation on Inhibition by Antisense
Phosphorothioate Oligodeoxynucleotides

30 To further define the effects of serum
preparation on the inhibitory activity of
phosphorothioate oligodeoxynucleotides, FBS that had been
heated to 56°C for 30 minutes, 68°C for 1 hour, or not

heated prior to addition to cultures was added to cultures of RS11846 lymphoma cells.

RS11846 cells were cultured in medium containing 1% (vol:vol) HL1-concentrate or 5% (vol:vol) FBS that had been heated at 56°C for 0.5 hour, 68°C for 1 hour, or that had not been heated. Cell counts were calculated as a percentage relative to control cultures treated with equivalent concentrations of TI-S phosphorothioate oligodeoxynucleotide, and represent the mean percentage (standard deviation was less than 10% for all values) for duplicate cultures counted on days 4 and 5.

The TI-AS phosphorothioate oligodeoxynucleotide completely inhibited the growth of RS11846 cells at 25 μ M, with an estimated half-maximal inhibitory concentration of approximately 11 μ M. In contrast, this phosphorothioate oligodeoxynucleotide was considerably less effective in cultures containing 5% (v:v) FBS. Furthermore, heating FBS prior to adding it to cultures did not significantly improve the ability of the TI-AS phosphorothioate oligodeoxynucleotide to suppress the growth of RS11846 lymphoma cells. At an oligodeoxynucleotide concentration of 50 μ M, inhibition of proliferation of RS11846 cells never exceeded 48% serum-containing cultures, regardless of the heating procedure used.

EXAMPLE 10

Influence of Dialysis of Serum on Inhibition by Normal and Phosphorothioate Antisense Oligodeoxynucleotides

To further characterize the nature of the interfering substances in serum, experiments were performed wherein 68°C-heated serum was extensively dialyzed (molecular weight cutoff = 3500) prior to being added to cultures of 697 leukemic cells. Experiments were conducted with 12.5 μ M TI-AS phosphorothioate

oligodeoxynucleotide and 200 μ M of the normal oxygen-based TI-AS oligodeoxynucleotide.

697 cells were cultured in medium containing 1% (vol:vol) HL1-concentrate (A) or 5% (vol:vol) of three different lots of 68°C-treated FBS (B,C,D). Each serum preparation was contrasted before (ND) and after (D) extensive dialysis. TI-AS (+) and TI-S (-) oligodeoxynucleotides were added to replicate cultures at 200 μ M for normal oxygen-based oligodeoxynucleotides (OXY) and at 12.5 μ M for phosphorothioate oligodeoxynucleotides (PT). Relative levels of DNA synthesis (kcpm) were measured after 2 or 4 days of culture for normal and phosphorothioate oligodeoxynucleotides, respectively.

For the three different lots of FBS tested, two exhibited little change after dialysis in cultures containing either normal or phosphorothioate oligodeoxynucleotides. One lot of FBS, however, appeared to interfere less with the inhibitory activities of these antisense oligodeoxynucleotides after dialysis.

EXAMPLE 11

Experiments with Stably Transfected NIH 3T3 Cells

Though the antisense oligodeoxynucleotides described herein were designed to block *bcl-2* mRNA translation (TI-AS) and splicing (SD-AS), the molecular mechanisms of their actions are not yet known. To determine the effect of formation of oligodeoxynucleotide-RNA hybrids within cells upon inhibition of cellular growth, irrespective of the nucleotide sequence, cells transformed to express human *bcl-2* cDNA transcripts were cultured with normal oligodeoxynucleotides.

200 μ M of normal TI-AS and TI-S
oligodeoxynucleotides were added to cultures of typical
NIH 3T3 cells and to cultures of these cells that had
been stably transfected with expression constructs that
5 produce high levels of human *bcl-2* cDNA transcripts for
either the usual sense (3T3-alpha-S cells) or the
antisense (3T3-alpha-AS cells) strand.

For RNA blot analyses, polyadenylated mRNA was
purified from normal NIH 3T3 cells and from cells stably
10 transfected with expression constructs that produce
either sense (3T3-alpha-S) or antisense (3T3-alpha-AS)
recombinant *bcl-2*-alpha mRNAs, according to the method of
13. Approximately 5 μ g of mRNA was subjected to RNA blot
analysis, essentially as described in (16), using either
15 3 _{2p}-labeled hybridization probes derived from human or
murine *bcl-2* sequences.

An autoradiogram resulting from a one-day
exposure of a blot containing RNAs from normal 3T3 cells,
3T3-alpha-AS cells, and 3T3-alpha-S cells showed high
20 relative levels of recombinant 2.4 and 1.4 kbp *bcl-2*
transcripts produced from the *bcl-2* expression constructs
that were transfected into 3T3-alpha-AS and 3T3-alpha-S
cells.

A 10-day exposure of a blot containing RNA from
25 normal 3T3 cells that were either proliferating or
quiescent at the time of harvesting RNA showed low but
detectable levels of normal 7.5 and 2.4 kbp murine *bcl-2*
transcripts present in proliferating 3T3 cells.

TI-AS oligodeoxynucleotide specifically
30 suppressed DNA synthesis and cellular replication in
cultures of normal NIH 3T3 cells, consistent with
findings by others that fibroblasts do contain *bcl-2*
transcripts, albeit at low levels. The TI-AS

oligodeoxynucleotide disclosed herein is complementary to the mouse *bcl-2* sequence in 18 of its 20 bases (17), accounting for its ability to suppress the growth of murine NIH 3T3 cells.

5 NIH 3T3 cells, 3T3-alpha-AS cells, and
3T3-alpha-S cells were cultured in medium containing 5%
(vol:vol) 68°C-treated serum and either HBSS, 200 μ M TI-S
normal oligodeoxynucleotide, or 200 μ M TI-AS normal
10 oligodeoxynucleotide. Relative levels of DNA synthesis
(kcpm) were measured in cultures after 3 days and reflect
a 16 hour incubation with 0.5 μ ci/well of [3 H]-thymidine.
Cell densities, estimated by phase microscopy, were
consistent with the measured DNA synthesis in cultures.
The percentage of inhibition of DNA synthesis in cultures
15 containing TI-AS oligodeoxynucleotides was calculated
relative to control cultures containing HBSS.

As with normal NIH 3T3 cells, culturing
3T3-alpha-S cells (producing human *bcl-2*-alpha sense
transcripts) with TI-AS and TI-S oligodeoxynucleotides
20 demonstrated specific suppression, since the sense
oligodeoxynucleotide TI-S was not inhibitory. The level
of inhibition of cellular proliferation by the antisense
oligodeoxynucleotide, however, was not as great in
3T3-alpha-S cells, as might be expected, since these
25 cells contain more *bcl-2* mRNA.

Adding TI-S oligodeoxynucleotide to cultures of
3T3-alpha-AS cells (produce antisense *bcl-2* transcripts)
ruled out inhibition of cellular growth through a
nonspecific mechanism involving oligodeoxynucleotide--RNA
30 hybrid formation. The TI-S oligodeoxynucleotide caused
little suppression of 3T3-alpha-AS cell proliferation,
whereas the TI-AS oligodeoxynucleotide was markedly
inhibitory in these cells. Similar data were obtained

with TI-AS and TI-S phosphorothioate oligodeoxynucleotides.

EXAMPLE 12

Measurements of DNA Fragmentation as an Indicator of 5 bcl-2 Antisense Oligodeoxynucleotide-Mediated Programmed Cell Death in Human Lymphoma Cells

Oligonucleotides having the sequences shown in Table 2 were tested for the ability to induce programmed cell death (DNA fragmentation) in the human
10 t(14:18)-containing human lymphoma cell line RS11846. The oligonucleotides were all phosphodiester, and were targeted against the translation initiation site or the 5'-cap region of bcl-2 pre-mRNAs. Control
15 oligodeoxynucleotides included a bcl-2 sense version (TI-S) of TI-AS (having SEQ ID NO: 7) and a scrambled version of TI-AS that has the same base composition, but with jumbled nucleotide order.

TABLE 2

	<u>SEQUENCE</u>	<u>SEQ ID NO:</u>
20	CGCGTGCGAC CCTCTTG	8
	TACCGCGTGC GACCCTC	9
	CCTTCCTACC GCGTGCG	11
	GACCCTTCCT ACCGCGT	12
	GGAGACCCTT CCTACCG	13
25	GCGGCGGCAG CGCGG	14
	CGGCGGGGCG ACGGA	15
	CGGGAGCGCG GCGGGC	16

RS11846 cells were adapted to grow in HL1 media with 1% FCS and their DNA metabolically labeled by
30 addition of ²⁵I-deoxyuridine to cultures for three hours. Labeled cells were then washed thoroughly and cultured for two days in the presence of various oligonucleotides at 50 AM. Cells were then recovered from 200 μL cultures by centrifugation, and lysed in a hypotonic buffer

containing 10 mM EDTA and 14 Triton X100. After centrifugation at 16,000 xg to pellet unfragmented genomic DNA, the supernatant fraction containing fragmented DNA was extracted with phenol/chloroform and ethanol precipitated. This DNA was then subjected to gel electrophoresis in 1.5% agarose gel and transferred to nylon membranes for autoradiography.

The results of two experiments are shown in Figures 3 and 4. The six *bcl-2* antisense oligonucleotides targeted in the vicinity of the ATG site of translation initiation in *bcl-2* mRNAs were tested. "C-Oligo-2" refers to an oligonucleotide with 4 purposeful mismatches. "U" indicates untreated control cells. Figure 4 shows the results for the oligonucleotides shown in Figure 3. "Sc20" refers to a 20 mer with the same base composition as TI-AS, but with scrambled sequence. Figure 4(b) shows the results for three oligonucleotides targeted against the 5'-cap of *bcl-2* mRNAs. The numbers refer to the distance of these oligomers from the ATG-translation initiation site.

The presence of a ladder of DNA fragments (unit size of approximately 200 bp) is indicative of programmed cell death. At 50 μ M, TI-AS caused little DNA fragmentation, whereas the oligonucleotides having SEQ ID NO: 9 and SEQ ID NO: 10, and one of the 5'-cap oligonucleotides (SEQ ID NO: 14) led to pronounced DNA fragmentation.

EXAMPLE 13

Concentration-Dependence of Inhibition by Antisense Phosphodiester Oligodeoxynucleotides in Serum-Free Cultures

697 pre-B cell leukemia cells were cultured in medium with either 1% (vol:vol) HL-1 concentrate (serum-free conditions [0] or 3% (vol:vol) 68°C-treated

serum (FBS2) [___], see Figure 5. Shown are cellular densities measured after 2 days in cultures containing various concentrations of phosphodiester TI-AS oligodeoxynucleotide. Data are shown as percentages relative to control cultures treated with a sense oligonucleotide, and reflect the mean \pm standard deviation for duplicate samples.

EXAMPLE 14

Immunofluorescence Analysis of bcl-2 Protein Levels in

10 Oligodeoxynucleotide-Treated 697 Cells

For studies with oligodeoxynucleotides, 0.25×10^4 (for phosphorothioate) or 0.5×10^5 (for normal oligodeoxynucleotides), 697 cells were cultured in 1 ml of HL-1 serum-free medium in 24 well culture dishes (Linbro. Flow Lab, Inc.). After 2 days (for normal) or 15 4 days (for phosphorothioates), cells were recovered from cultures, washed once in [PBS, pH 7.4 (Gibco) - 0.1% bovine serum albumin - 0.1% sodium azide], and fixed for 5-10 minutes on ice in 1% paraformaldehyde/PBS solution. 20 The cells were then washed once in PBS and incubated in 1 ml of absolute methanol at 20°C for 10 minutes. After washing once in PBS-A, cells were then resuspended in PBS containing 0.05% Triton-X100 for 3 minutes on ice, washed in PBS-A and preblocked for 30 minutes at 4°C in PBS with 25 10% (v/v) heat-inactivated goat serum.

For addition of the first antibody, preblocked cells were resuspended in 100 μ l of PBS-G (PBS-1% goat serum-0.1% sodium azide) prior to aliquoting 50 μ l into separate tubes that contained 1 μ l of either BCL2 30 antibody (Halder et al., Nature (London), 342:195-197 (1989)) or affinity-purified normal rabbit control IgG (Cappel 6012-0080) and incubated for 1 hour on ice. The BCL2 antibody used for these studies was prepared in rabbits using a synthetic peptide corresponding to amino 35 acids (98-114) of the BCL2 protein and was affinity--

purified by protein-A-Sepharose chromatography and used at approximately 1 mg/ml. Cells were then washed in PBS-A and incubated in 0.5-1.0 ml PBS-A for 15-20 minutes on ice to allow diffusion of nonspecific cell-associated antibody prior to resuspending cells in 100 μ l of PBS-G containing 5 μ g of biotinylated scat anti-rabbit IgG (BAI000; Vector Labs) for 30 minutes. After washing once and incubating for 15 minutes in PBS-A, cells were finally resuspended in 100 μ l of PBS-A containing 2 μ g of FITC-conjugated avidin (Vector Labs A2011) for 20 minutes and washed three times in PBS-A prior to analysis with an Ortho cytofluorograph 50-H connected to an Ortho 2150 data-handling system. The specificity of method for detecting BCL2 protein was confirmed by immunofluorescence microscopy (showing cytosolic stain peptide competition, and studies of cell lines that expressed various levels of BCL2 mRNA and proteins through gene transfer manipulations.

For measurements of surface HLA-DR antigen expression, an indirect immunofluorescence assay method was used (Reed et al., *J. Immunol.* 134:1631-1639 (1985)) involving incubation of viable cells with a murine anti-HLA-DR monoclonal antibody (IgG2a) (Becton-Dickinson 7360) or a negative control antibody, R3-367 (IgG2a), followed by FITC-conjugated scat anti-mouse IgG (Cappel 1711-0081). Cells were fixed in 1% paraformaldehyde/PBS prior to FACS analysis.

697 cells were cultured for 2 days (PO) or 4 days (PS) with various oligonucleotides. In Figure 6, the black columns show the results with a sense oligonucleotide, and the hatched columns with an antisense oligonucleotide TI-AS. Cells were labeled with anti-bcl-2 antiserum and analyzed by FACS. Data are expressed as percentages relative to the mean fluorescence obtained with untreated 697 cells.

Figure 7 shows typical FACS results obtained for 697 cells before and after treatment with 100 μ M PO *bcl-2* antisense oligonucleotides. A: untreated 697 cells labeled with either anti-*bcl-2* antiserum (hatched area) or normal rabbit serum control (white area); B: untreated 697 cells labeled with either anti-HLA-DR antibody (hatched area) or a negative control antibody (white area); C: 697 cells cultured for 2 days with either normal *bcl-2* TI-AS (white area) or TI-AS (hatched area) oligodeoxynucleotides and labeled with anti-*bcl-2* antibody; D: 697 cells cultured with TI-AS and TI-S oligodeoxynucleotides (as in C), but labeled with anti-HLA-DR antibody.

As shown in Figures 6 (a) and (b) , PO and PS *bcl-2* antisense oligonucleotides produced specific concentration-dependent reductions in the levels of *bcl-2* proteins, without altering the levels of expression of HLA-DR (Figure -7) and other control antigens. At 150 μ M, for example, PO antisense oligodeoxynucleotide caused an approximately 75-95% reduction in *bcl-2* fluorescence, whereas the control sense oligodeoxynucleotide diminished *bcl-2* protein levels by only 10-20% (Figure 6(a)). Similarly, cultured 697 cells for 4 days with the PS antisense oligodeoxynucleotide at 25 μ M resulted in approximately 70% reduction in *bcl-2* fluorescence. In comparison, the sense PS oligodeoxynucleotide TI-AS inhibited *bcl-2* protein levels by only approximately 15%, as measured by this assay (Figure 6(b)).

SIGNIFICANCE

In phosphorothioate oligodeoxynucleotides, one of the non-bridging oxygen atoms in each internucleotide phosphate linkage is replaced by a sulfur atom. This modification renders phosphorothioate oligodeoxynucleotides extremely resistant to cleavage by nucleases, Stein et al., *Nucl. Acids Res.*, 16:3209-3221

(1988). Despite the substitution of a sulfur atom for an oxygen, phosphorothioate oligodeoxynucleotides retain good solubility in aqueous solutions; hybridize well, though with some decrease in the melting temperature of RNA-oligodeoxynucleotides duplexes; and are synthesized conveniently by the widely employed method of automated oligodeoxynucleotides synthesis with phosphoroamidites.

Antisense *bcl-2* phosphorothioate oligodeoxynucleotides have been found to be more potent inhibitors of leukemic cell growth than their normal oxygen-based counterparts. When tested under serum-free conditions, these oligodeoxynucleotides reduced cellular proliferation by half at concentrations of approximately 15-23 μM , whereas the normal oligodeoxynucleotide achieved 50% inhibition at 125-250 μM . This finding may be explained by the reduced sensitivity of phosphorothioate oligodeoxynucleotides to cellular nucleases, or may be attributable to other mechanisms. For example, mRNAs hybridized with phosphorothioate oligodeoxynucleotides may experience enhanced degradation through a mechanism involving an RNase H-like activity.

Despite their increased inhibitory activity, phosphorothioate antisense oligodeoxynucleotides retained sequence-specificity. At the concentrations tested (less than 25 μM), sense versions of these oligodeoxynucleotides had little effect on leukemic cell growth. Both normal and phosphorothioate antisense oligodeoxynucleotides appeared to initially suppress the proliferation of leukemic cells through non-cytotoxic mechanisms. During the first few days of culture, cellular replication was inhibited without a concomitant rise in cell death. Later in these cultures (days 4-5 for normal oligodeoxynucleotides, days 6-8 for phosphorothioates), however, cellular viabilities declined.

Comparing the kinetics of inhibition by normal and phosphorothioate oligodeoxynucleotides revealed that the latter compounds have a slower onset of action.

5 Maximal inhibition of leukemic cell proliferation by normal antisense oligodeoxynucleotides occurred two days after initiation of cultures, whereas phosphorothioate oligodeoxynucleotides required 4 to 7 days to achieve maximal inhibition.

10 The usefulness of anticod oligomers in inhibiting human lymphoma/leukemia cells and other types of cancer cells that express the *bcl-2* gene has been shown by the examples herein. Anti-sense oligodeoxynucleotides complementary to at least an effective portion of the mRNA of the human *bcl-2* gene has
15 been found to inhibit growth of RS11846 human follicular lymphoma cells t (14;18) chromosomal translocation and high *bcl-2* expression), 697 human pre B cell leukemia cells (high *bcl-2* expression), JURKAT human acute lymphocytic leukemia cells (medium *bcl-2* expression),
20 normal human lymphocytes (medium *bcl-2* expression) and murine fibroblasts (low *bcl-2* expression). Although *bcl-2* antisense reagents can suppress the growth of many types of cells, the t(14;18) lymphoma and leukemia cells seem to be the sensitive, allowing for specific
25 inhibition of malignant cells.

As demonstrated in the following Examples, a variety of DNA analogs can be employed in the instant invention. For example, phosphorothioates, methylphosphonates, and mixed oligomers containing
30 combinations of phosphodiester and phosphorothioate or methylphosphonate nucleosides. It should be understood that RNA analogs can also be employed in the invention.

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EXAMPLE 15

Methylphosphonate (MP)/Phosphodiester (PO) bcl-2
Antisense Oligomers Induce Death of DoHH2 Lymphoma Cells

5 The purpose of this study was to determine the
efficacy of various analogs of the anticode oligomers for
inhibiting lymphoma cell survival.

DoHH2 is a human lymphoma cell line containing
a t(14:18)-translocation that activates the bcl-2 gene.
DoHH2 cells were cultured for 3 days without oligomers or
10 in the presence of various concentrations of antisense
(As) and scrambled (Sc) methylphosphonate
(MP)/Phosphodiester (PO) oligomers for 3 days. Cell
viability was assessed by trypan blue dye exclusions, and
the data expressed as a percentage relative to DoHH2
15 cells cultured without oligomers. The MP/PO oligomers
was an 18-mer targeted against the first 6 codons of the
bcl-2 open reading frame in which 5 internal linkages
were phosphodiester and the flanking nucleosides were
methylphosphonates.

20 The results indicate that these anticode
oligomer analogs are potent and specific inhibitors of
lymphoma cell survival.

EXAMPLE 16

Methylphosphonate (MP)/Phosphodiester (PO) Chimeric
25 Oligomers Inhibit Growth of MCF-7
Human Breast Cancer Cells

The purpose of this study was to determine the
efficacy of the claimed anticode oligomer analogs to
inhibit the survival of solid tumor cells which highly
30 express bcl-2.

MCF-7 is a human breast adenocarcinoma cell line
that contains relatively high levels of bcl-2 protein.
The cells were cultured at 4,000 cells per well in 96-

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well microtiter plates in the presence or absence of MP/PO oligomers. Relative cell numbers per well were then estimated by MTT assay, based on a standard curve prepared using freshly plated, untreated MCF-7 cells.

5 The antisense (As) and scrambled (Sc) MP/PO oligomers were the same as those described in Example 16. Data represent the mean \pm standard deviation for determinations.

The results demonstrate sequence specific inhibition of growth of solid tumor cells by the claimed anticode oligomer analogs.

EXAMPLE 17

Optimization of Anticodone bcl-2 Oligomer Sequences

60 The purpose of this study was to determine optimum target sites or sequence portions on mRNA for inhibiting cell survival by contacting the cells with various claimed anticodone molecules whose sequences were computer generated.

DoHH2 lymphoma cells were treated with various concentrations of oligomers targeted to different sites on the bcl-2 mRNA. The ATG oligomer targets the translation initiation site, and is complementary to the first 6 codons of the open reading frame. The Dscore 23 and Dscore 72 oligomers target sites in the 5' untranslated region of the mRNA. Sc oligomers represent negative controls having the same length and base composition but in scrambled order. All oligomers were prepared as phosphodiester (PO)/phosphorothioate (PS) chimeras, where only the last (3') two internucleoside linkages were phosphorothioates. Oligomers were added directly to cultures and relative numbers of viable cells were estimated by MTT assay 3 days later. Data represent mean \pm standard deviation.

The results indicate that the Dscore 23 oligomer, targeted to the 5' untranslated region, has, compared to the other anticode oligomers tested in this Example, superior activity for inhibiting cell survival.

5

EXAMPLE 18

Reveral of Chemoresistance of Tumor Cells by Antisense-Mediated Reduction of bcl-2 Gene Expression

The following work was undertaken to determine if anticode oligomers directed against the expression of the bcl-2 gene would reverse chemoresistance, that is to say, increase the sensitivity to cancer chemotherapeutic agents in cancer tumor cells expressing the bcl-2 gene.

High levels of bcl-2 protein appeared to increase the relative resistance of lymphoid cells to killing induced by a wide variety of cancer chemotherapeutic agents including, but not limited to, Ara-C, MTX, vincristine, taxol, cisplatin, adriamycin, etoposide, mitozantron, 2-chlorodeoxyadenosine, dexamethasone (DEX), and alkylating agents. (Miyashita, T. and Reed, J.C., Cancer Res. 52:5407, October 1, 1992). While these drugs have diverse biochemical mechanisms of action, it is believed that all have in common the ability to ultimately trigger cancer cell death by activating endogenous cellular pathways leading to apoptosis (Eastman, A. Cancer Cells 2:275 (1990)). It is understood that the claimed anticode molecules and analogs thereof as used herein are effective for their intended purposes of enhancing sensitivity to cancer chemotherapeutic drugs including, but not limited to, antimetabolites, alkylating agents, plant alkaloids, and antibiotics.

5 Alkylating agents include, but are not limited to, cyclophosphamide, melphalan, busulfan, cisplatin, paraplantin, chlorambucil, and nitrogen mustards.

10 Antibiotics include, but are not limited to,
doxorubicin (adriamycin), daunorubicin, mitomycin c,
bleomycin.

Other cancer chemotherapeutic agents include DTIC (decarbazine), mAMSA, hexamethyl melamine, mitroloxantrone, taxol, etoposide, dexamethasone.

In the present work, both nuclease resistance phosphorothioates (PS) and phosphodiester in which only the 3'-most internucleoside bond was a thioate linkage (PO/PS) were employed. The PO/PS oligomers are resistance to 3' exonucleases (the principal nuclease activity of serum) and generally form more stable heteroduplexes with target RNAs.

Cationic lipids were used to improve the uptake and subsequent release of oligomers into effective intracellular compartments, and are exemplary pharmaceutical carriers for the claimed anticode oligomers.

The methods for preparing and purifying the antisense (AS) and scrambled (SC) 13'mer oligonucleotides used for the present work are described above in General

Methods and in Kitada et al. (Antisense R & D, 3:157 (1993)). Phosphodiester oligonucleotides were synthesized in a 10-15 micromole scale using phosphoroamidate chemistry with oxidation by iodine, and then purified using a C₁₈-reverse phase column. In most cases, oligomers were additionally ethanol-precipitated five times to eliminate any nonspecific cytotoxic activity, and then dried and resuspended in sterile HL-1 medium (Ventrex Labs, Inc; Burlingame, CA) at 1-10 mM. The pH of this solution was adjusted using 1-10 M NaOH until the phenol red indicator dye in the media returned to its original color.

The principal oligomers used were 18-mers, having either the sequence:

- I. TCTCCCAGCGTGGCCAT (SEQ ID NO. 17), which is antisense to the first six codons of the human bcl-2 open reading frame (SEQ ID NO. 19); or
- II. TGCACTCAGCTCGGCCT (SEQ ID NO. 18), which is a scrambled version used as a control.

Standard transfection methods were used to produce tumor cells expressing either the bcl-2 gene or an antisense oligodeoxynucleotide which bound to bcl-2 mRNA. It is understood that the vector could also encode an antisense oligodeoxynucleotide which binds to bcl-2 pre-mRNA. The particular nucleotide sequence encoding the antisense oligonucleotides of the invention is not critical, except that the sequences are preferably chosen such that they express antisense oligodeoxynucleotides sufficient to reduce bcl-2 gene expression in tumor cells and increase the sensitivity of the tumor cells to cancer chemotherapeutic agents or sufficient to kill tumor cells when they are treated with cancer chemotherapeutic agents. It is only necessary that the antisense oligodeoxynucleotide encoded in vector is expressed under

conditions sufficient to reduce bcl-2 gene expression in tumor cells. The methods used for preparing vectors, and, in particular, expression plasmids, for transferring genes into mammalian cells relies on routine techniques in the field of molecular biology. A basic text disclosing the general methods of preparing expression plasmids used in this invention is Molecular Cloning, A Laboratory Manual, 2nd Edition, eds. Sambrook et al., Cold Spring Harbor Laboratory Press, (1989), particularly Chapter 16 on Expression of Cloned Genes in Cultured Mammalian Cells. Examples 15C-D below set forth particular methods for preparing the expression plasmids used in the present invention. The particular vector used to transfer the antisense oligonucleotides of the present invention is not critical, and such vectors may include vectors derived from lambda and related phages or from filamentous phages. It is only necessary that the transferred nucleotide sequence encoding the antisense oligonucleotides of the present invention be expressed in the transfected tumor cell under conditions sufficient to reduce bcl-2 gene expression in the tumor cell. The present invention includes expression of the antisense oligonucleotide either from an extrachromosomal position (e.g. from an expression plasmid) or from a position integrated into the host genome itself, as mediated by other vectors, such as recombinant retroviral vectors (Reed et al. bcl-2 mediated tumorigenicity in a T-cell lymphoid cell line: synergy with C-MYC and inhibition by bcl-2 antisense. PNAS USA 87:3660 (1990)).

A. Treatment of Lymphoma Cells With 18-mer Synthetic bcl-2 Antisense Oligodeoxynucleotides.

Lymphoma cell line SU-DHL-4, obtained from a use of diffuse, histiocytic, non-Hodgins lymphoma (Epstein et al. Two new monoclonal antibodies (LN-1, LN-2) reactive in B5 formalin-fixed, paraffin-embedded

tissues with follicular center and mantle zone human B lymphocytes and derived tumors. J. Immunol. 133:1028 (1984)) and containing a t(14;18) translocation was treated with 18-mer synthetic bcl-2-AS
5 oligodeoxynucleotides targeted for binding with the first six codons of the bcl-2 mRNA. As a control, SU-DHL-4 cells were treated with various control oligomers, including 18-mers having the same nucleoside composition as the AS oligomer, but in which the bases were in
10 scrambled order (SC).

Aliquots of 1.5 ml of HL-1 serum-free medium (Ventrex Labs, Inc.) supplemented with 1 mM L-glutamine, 50 Units/ml penicillin, and 100 ug/ml streptomycin and either 5 ug of purified oligonucleotides or 30 ug of
15 Lipofectin[®] [1:1 w/w mixture of N-(1-2,3-dioleoyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoylphosphatidylethanolamine (DOPE)] were combined and added to 0.75 X 10⁶ SU-DHL-4 cells in 3 mls of HL-1 medium. Cells were then either
20 cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air in 24 well plates (2 mls/well) for immunoblot and RT-PCR assays, or in 96-well flat-bottom microtiter plates (0.1 ml/well) for MTT assays. For cells in microtiter cultures, typically 0.1 ml of additional HL-1
25 media with or without various chemotherapeutic drugs was added after 1 day, and the cells were cultured for an additional 2 days before performing MTT assays.

Cells were washed once in PBS, lysed in a buffer containing 1% Triton X100, and samples normalized
30 for protein content (25 ug) prior to size-fractionation of proteins by SDS-PAGE (12% gels) and transfer to nitrocellulose filters for immunoblot assays as described in Reed et al. Cancer Res. 51:6529 (1991). Preliminary experiments determined that aliquots of lysates
35 containing 25 ug of total protein produced results in the

linear range of the assay. Blots were first incubated with 0.1% (v.v) of a rabbit antiserum directed against a synthetic peptide corresponding to amino-acids (aa) 41-54 of the human Bcl-2 protein, as shown in SEQ ID NO. 21 (id) followed by 2.8 ug/ml biotinylated goat anti-rabbit IgG (Vector Labs, Inc.). Bands corresponding to p26-Bcl-2 were then visualized by color development using a Horseradish Peroxidase (HRP)-avidin-biotin complex reagent (Vector Labs, Inc) and 3,3'-diaminobenzidine (DAB). Stained blots were then incubated with a second anti-Bcl-2 antibody directed against aa 61-76 of the Bcl-2 protein (SEQ ID NO. 21) followed by 0.25 uCi/ml ¹²⁵I-protein A. Bcl-2 bands were excised from the blots and subjected to gamma-counting.

Despite the mitochondrial location of Bcl-2 protein, no difference in the rate of MTT dye reduction by mitochondrial enzymes was noted in cells that were identical except for their levels of p26-Bcl-2. These comparisons were made using pairs of exponentially growing lymphoid cell lines that differed only in that one line had been stably infected with a recombinant bcl-2 retrovirus and the other with the parental retroviral vector lacking a bcl-2 cDNA insert (Miyashita et al. Cancer Res. 52:5407 (1992); Blood 81:151 (1993)).

Anticodone specific reductions in the relative levels of bcl-2 mRNA were detected within 1 day by a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. See Figure 8A.

SU-DHL-4 cells were cultured with 0.83 ug/ml of oligomers complexed with 5 ug of cationic lipids (Lipofectin; BRL/Gibco, Inc.) per ml of serum-free media (13,19). In Figure 8A, total RNA was isolated from cells after 1 day and relative levels of bcl-2 and glyceraldehyde 3'-phosphate dehydrogenase (GAPDH) mRNAs

were assessed by RT-PCR assay as described in Kitada et al. Antisense R & D 3:157 (1993)).

In Figure 8B, SU-DHL-4 cells were cultured with pairs of either PS (squares) or PO/PS (circles) As- and Sc-oligomers for 3 days. Relative levels of Bcl-2 protein were then measured using a quantitative immunoblot assay, as described above, and the data expressed as a percentage relative to cells treated with control Sc-oligomers. The inset shows immunoblot results for p26-Bcl-2 and a p75 cross-reactive (CR) band in a case where As-PO/PS oligomer produced a 41% relative decrease in Bcl-2 protein levels. In Figure 8C, 10^{-4} M Ara-C, MTX, or DEX was added 1 day after addition of PS (squares) or PO/PS (circles) oligomers to cultures of SU-DHL-4 cells, and MTT assays were performed on day 3. Data are presented as a % control relative to cells cultured with drugs in the absence of any oligomers, and represent the results of 9 of 10 consecutive experiments [in one experiment, the MTT assay failed]. Similar results were obtained when dye exclusion assays were used to assess cell survival rather than MTT assay [not shown].

Mean values for the data are indicated by horizontal lines. Statistical analysis of the data was by paired t-test (As versus Sc). Concentrations of As- and Sc-oligomers (≈ 150 nM) were adjusted to maximize As effects while maintaining sequence specificity.

Variations in the amounts of starting RNA were controlled for by RT-PCR analysis using primers specific for GAPDH mRNA.

The long half-life of the bcl-2 protein (approximately 14 hours) may account for the AS-mediated reductions in bcl-2 proteins not being as dramatic as for

reductions in bcl-2 mRNA, taking longer to achieve (about 3 days), and appearing more variable.

Figure 8B shows the composite results for 10 experiments where relative levels of bcl-2 protein were compared in SU-DHL-4 cells treated with AS or SC oligomers. AS-mediated reductions in bcl-2 protein levels ranged from as much as 66% to as little as 10%, with an average relative reduction of about 30%, compared to SU-DHL-4 cells that were treated in the identical manner with control oligomers. Levels of a variety of control mitochondrial proteins such as F₁-beta-ATPase and cytochrome C, which like bcl-2 are encoded by nuclear genes, were not adversely affected by AS-oligomers (not shown), indicating that the AS-mediated reductions in bcl-2 protein levels were specific. The insert in Figure 8B, for example, shows a comparison of p26-Bcl-2 with a 78-kDa protein that cross reacts with one of the rabbit antisera employed for immunoblot assays, demonstrating a decrease in the levels of p26-bcl-2 but not p78 in the AS-treated cells relative to cells that received control SC-oligomers.

B. Effect of Treatment of SU-DHL-4 Cells with bcl-2 AS Oligomers on Cell Sensitivity to Cancer Chemotherapeutic Agents

This study was performed to determine whether treatment of SU-DHL-4 cells with bcl-2 AS-oligomers could increase their relative sensitivity to killing by the cancer chemotherapeutic agents Ara-C, MTX, and DEX, which are anticancer drugs.

Previous control studies demonstrated that bcl-2 AS oligomers had little or no effect on SU-DHL-4 cell growth and survival at least during the first three days of culture (Kitada et al. Antisense R & D 3:157 (1993)). AS-mediated reductions in bcl-2 protein levels in these

lymphoma cells as well as in other cells do not typically accelerate the rate of cell death in cultures unless the cells are deprived of serum growth factors (Reed et al. Proc. Natl. Acad. Sci. USA 87:3660 (1990)).

5 In the present work, preliminary studies
demonstrated that more than 90% of SU-DHL-4 cells
survived treatment for 4 days with high dose (10^{-4}) Ara-C,
MTX or DEX, presumably because of their high levels of
10 bcl-2 protein (Not shown). At these concentrations,
however, all drugs induced essentially complete
inhibition of SU-DHL-4 cell proliferation, consistent
with bcl-2 converts drugs from cytotoxic to cytostatic.
Comparisons of AS and SC oligomers demonstrated that bcl-
2 AS treatment markedly enhanced the sensitivity of these
15 lymphoma cells to MTX and Ara-C, and to a lesser extent
to DEX (Figure 3C).

 Despite some variability in results, on
average, the addition of bcl-2 AS oligomers to cultures
of SU-DHL-4 cells treated with MTX or Ara-C resulted in
20 79-84% greater inhibition (reduction in viable cell
numbers) than use of either drug alone ($P < 0.002$ for AS
versus SC) in the absence of introducing the bcl-2 AS
oligomers of the invention. Statistically significant
results were obtained for DEX-treated SU-DHL-4 cells
25 ($P=0.01$). The 20-30% reduction in viable cell numbers
observed for control oligomer-treated cells could reflect
a degree of sequence non-specificity, but was probably
related to the use of cationic lipids to facilitate
oligomer delivery into cells.

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C. Effect of Transfecting Cells With Expression Plasmids Encoding Human bcl-2 Protein on Sensitivity to Chemotherapeutic Agents.

5 To further confirm the sequence specificity of
bcl-2 AS oligomers for enhancing sensitivity to
chemotherapeutic anticancer drugs, a study was conducted
using an Interleukin-3 (IL-3)-dependent murine
hemopoietic cell line 32D.C13 that had been stably
transfected with expression plasmid encoding either the
10 human bcl-2 protein or a viral homolog of bcl-2, BHRF-1,
which has only 22% homology with bcl-2. 32D.C13 cells
were obtained from Dr. Giovanni Rovera of the Wistar
Institute, Philadelphia, PA.

15 Treatment of 32D cells with oligomer/cationic
lipid complexes was as described above except that 50
Units/ml of murine recombinant IL-3 (rIL-3) was included
in the HL-1 media, the initial cell density was 10^5 per
ml, and replication-defective adenovirus dl312 (MOI=200)
was added 30 minutes after exposure of cells to oligomers
20 to facilitate exit of DNA from endosomes [Yoshimura K, et
al. J Biol Chem. 268, 2300, (1993)].

32D cells that had been stable transfected with
expression plasmids encoding either human p26-Bcl-2 or
EBV p19-BHRF-1 (Takayama, S. et al. submitted) were
25 cultured in medium (10^5 /ml) containing IL-3 and PO/PS
oligomers for 3 days to achieve reductions in human Bcl-2
protein levels. The cells were then retreated with
oligomers alone (C) or in combination with various
concentrations of MTX and the relative number of viable
30 cells assessed by MTT assay 2 days later. Data represent
mean +/- standard deviation for triplicate determinations
and are expressed as a % relative to cells that received
no MTX. Statistical analysis of data for 10^{-6} to 10^{-4} M
MTX was by a 2-way Analysis of Variables method (Finney,
35 D.J. In Statistical Methods in Biological Assays, p. 72,

1978 (3rd edition, Charles Griffin & Co., London).
Comparable results were obtained with dye exclusion
assays [not shown].

5 RNAs derived from the human bcl-2 construct in
32D-BCL-2 cells were a target for bcl-2 AS oligomers,
whereas RNAs from the BHRF-1 expression plasmid are not.
Thus the chemosensitivity to cytotoxic drugs of 32D.C13
cells expressing BHRF-1 should have been unaffected by
the AS treatment.

10 Preliminary experiments demonstrated that upon
withdrawal of IL-3 from 32D.C13 cells, levels of
endogenous mouse bcl-2 protein declined and the cells
underwent apoptosis. bcl-2 and BHRF-1 comparably
supported the survival of 32D.C13 cells in the absence of
15 IL-3, and the proliferative rates of 32D.C13 cells
containing high levels of these proteins were similar in
the presence of IL-3, thus excluding these variables as
explanations for any differences in chemosensitivity.

20 Figure 9 compares the sensitivity of 32D-BCL-2
and 32D-BHRF-1 cells to various concentrations of MTX.
Treatment with bcl-2 AS-oligomers resulted in sequence-
specific increases in the sensitivity of 32D-BCL-2 cells
to inhibition by MTX at concentrations of 10^{-5} to 10^{-6} M
($P \leq 0.001$ for AS versus SC). In contrast, treatment with
25 bcl-2 AS oligomers produced no significant difference in
the sensitivity of 32D-BHRF-1 cells to MTX, relative to
control SC-oligomers (Figure 9). These data indicate
that the effects of bcl-2 AS oligomers on
chemosensitivity to cytotoxic agents drugs are sequence
30 specific. Furthermore, several other control oligomer,
including bcl-2 sense, other scrambled sequences with the
same nucleoside composition as AS, and oligomers with
totally unrelated sequences all had comparatively little
effect on the chemosensitivity of the cells (Not shown).

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supplemented with 10% fetal bovine serum, 1 mM L-glutamine, 50 Units/ml penicillin, and 100 ug/ml streptomycin) at 2×10^5 cells per ml and cultured for 2 days before seeding cells at 2×10^5 per ml in media containing 200 ug/ml hygromycin. After 3 weeks of culture, the resulting bulk cell lines were passaged in successively higher concentrations of hygromycin in 200 ug/ml increments until the concentration reached 1 mg/ml (about 4 weeks).

Hygromycin-resistant RS11846 cell were cultured in RPMI/10% serum media containing 0.5 uM CdCl₂ and 3 days later immunoblot assays were performed using 25 ug protein/lane essentially as described in Tanaka S, et al. J. Biol. Chem. 268, 10920 (1993) and in Reed et al. Cancer Res. 51:6529 (1991)).

As summarized in Figure 10, control ("C") and *bcl-2*-As ("As") plasmids were introduced into RS11846 cells and expression was induced with either 0.5 uM CdCl₂ or 50 uM ZnCl₂ for various times. As an additional control, RS11846 cells containing inducible plasmids with the *bcl-2* cDNA in sense ("S") orientation were also analyzed. RS11846 cells were induced for 3 days and relative levels of Bcl-2 and F₁- β -ATPase proteins were assessed by immunoblot assay of Tanaka et al. J. Biol. Chem. 268:10920 (1993). In Figure 10A, RS11846 cells were cultured at 10^5 cells/ml in medium containing 0.5 uM CdCl₂ and 1 day later 10^{-7} M Ara-C or an equivalent volume of diluent control was added. Relative numbers of viable cells were estimated from MTT assays at various times and the mean \pm S.D. calculated for triplicate samples. In Figure 10B, RS11846 cells were cultured as in Figure 10A, except that various concentrations of Ara-C, MTX, or DEX were added. Data represent mean \pm S.D. for triplicate samples. Statistical calculations are by 2-way Analysis

of Variables. DEX served as a negative control here since RS11846 cells have lost glucocorticoid receptors.

Preliminary experiments demonstrated that RS11846 cells tolerated the addition of up to 0.5 microM CdCl₂ or to microM ZnCl₂ to cultures for one week, experiencing a slight decrease in growth rate but essentially no decline in percentage cell viability (Not shown).

In the absence of heavy metal induction, the relative levels of bcl-2 protein in RS11846 cells containing the control or bcl-2 AS plasmid were comparable, as determined by immunoblot assays (Not shown). When 0.5 uM CdCl₂ or uM ZnCl₂ was added, reductions in bcl-2 protein became evident in the AS-expressing cells at 2 days and maximal inhibition of 30-40% was obtained at three to four days, relative to control RS11846 cells.

Figure 10A shows an example of immunoblot data derived from RS11846 cells after three days of exposure to 0.5 mM CdCl₂, demonstrating reduced levels of bcl-2 protein in the AS-plasmid containing cells compared to RS11846 cells that harbored the control plasmid. The relative levels of a control mitochondrial protein F₁-beta-ATPase were comparable in all cell lines, consistent with sequence-specific alterations in bcl-2 protein levels.

When RS11846 cells containing either the control or bcl-2-As plasmids were cultured for various times in 0.5uM CdCl₂ or 50 uM ZnCl₂, no significant difference in the growth rates of these two cell lines was observed (Figure 8B). Thus, As-mediated reductions in Bcl-2 protein levels by themselves did not impair RS11846 cell proliferation or survival.

Inclusion of low-dose Ara-C (10^{-7} M) in the cultures of control RS11846 cells resulted in only a slight decline in the net numbers of viable cells, presumably because of the high levels of Bcl-2 protein found in these t(14;18)-containing lymphoma cells. In contrast, addition of 10^{-7} M Ara-C to cultures of *bcl-2*-AS expressing RS11846 cells was markedly inhibitory (Figure 8B). Ara-C, however, had no effect on *bcl-2* AS-expressing RS11846 cells in the absence of heavy metal induction of the MT promoter, when directly compared with RS11846 cells containing the control plasmid under the same conditions [not shown]. Figure 8C shows that the enhanced sensitivity to Ara-C observed for *bcl-2*-AS-expressing RS11846 cells occurred over a wide range of drug concentrations ($P < 0.001$). Heavy-metal induction of the *bcl-2*-AS expression plasmid also significantly increased the relative sensitivity of RS11846 lymphoma cells to MTX ($P < 0.001$), but not to DEX. Glucocorticoid receptor binding assays demonstrated that RS11846 cells have lost receptors for these steroid hormones [not shown], thus providing a specificity control showing that AS-mediated reductions in *bcl-2* protein levels are by themselves insufficient to impair the growth or survival of these lymphoma cells.

Using a plurality of anticode approaches, the present invention demonstrated that average reductions of 30-40% in the relative levels of *bcl-2* protein markedly enhanced the sensitivity of lymphoma cells, in particular, t(14;18)-containing lymphoma cell lines to chemotherapeutic agents such as conventional anticancer drugs. These examples demonstrated that introducing the claimed anticode oligomers into tumor cells achieves a reduction of *bcl-2* expression and increases the chemosensitivity of neoplastic cells to chemotherapeutic agents or anticancer drugs.

Accordingly, the present invention achieved a method of killing tumor cells by introducing to tumor cells anticode oligomers which reduce *bcl-2* gene expression or impair Bcl-2 protein function before
5 contacting the cells with chemotherapeutic agents including anticancer drugs. The conventional anticancer drugs reduced the numbers of viable malignant cells, and the portion of tumor cells killed was greater than the
10 portion which would have been killed by the same amount of drug in the absence of introducing the anticode oligomers into the cells.

Having thus disclosed exemplary embodiments of the present invention, it should be noted by those skilled in the art that this disclosure is exemplary only
15 and that various other alternatives, adaptations, and modifications may be made within the scope of the present invention. Accordingly, the present invention is not limited to the specific embodiments as illustrated herein, but is only limited by the following claims.

00375644 004790

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Reed, John
- (ii) TITLE OF INVENTION: Regulation of bcl-2 Gene Expression
- (iii) NUMBER OF SEQUENCES: 23
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Gray, Cary, Ames & Frye
 - (B) STREET: 401 B Street, Suite 1700
 - (C) CITY: San Diego
 - (D) STATE: CA
 - (E) COUNTRY: USA
 - (F) ZIP: 92101-4297
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette 3.5 inch, 1.44 Mb storage
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Brotman, Harris F.
 - (B) REGISTRATION NUMBER: 35461
 - (C) REFERENCE/DOCKET NUMBER: P0041US0
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (619) 699-3630
 - (B) TELEFAX: (619) 236-1048

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iv) ANTI-SENSE: YES
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCTTCCTAC CGCGTGCGAC

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTTCCTCT GGGAAGGATG GCGCACGCTG GGAGA

35

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCTCCGACCC ATCCACGTAG

20

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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33

664450 445260

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTGACGTCC TACGGAAACA

20

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 36 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCCCAACTG CAGGATGCCT TTGTGGAAC TGTACGG

36

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GGGAAGGATG GCGCACGCTG

20

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCGTGCGAC CCTCTTG

17

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TACCGCGTGC GACCCTC

17

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCCTACCGCG TGCGACC

17

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid

00354 05799
004750 059460

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCTTCCTACC GCGTGCG

17

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACCCTTCCT ACCGCGT

17

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGAGACCCTT CCTACCG

17

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

CCCTTCCTACC GCGTGCG

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCGGCGGCAG CGCGG

15

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGGCGGGGCG ACGGA

15

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGGGAGCGCG GCGGGC

16

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

CGGGAGCGCG GCGGGC

TCTCCCAGCG TGCGCCAT

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

TGCACTCACG CTCGGCCT

18

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 5086 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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GGGTGCGCCG GTGGGGTGCA GCGGAAGAGG GGGTCCAGGG GGGAGAACTT CGTAGCAGTC	300
ATCCTTTTTTA GGAAAAGAGG GAAAAAATAA AACCCTCCCC CACCACCTCC TTCTCCCCAC	360
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CAATACTTAC TAATAATAAC GTGCCTCATG AAATAAAGAT CCGAAAGGAA TTGGAATAAA	1260
AATTCCTGCT GTCTCATGCC AAGAGGGAAA CACCAGAATC AAGTGTTCCG CGTGATTGAA	1320

00354 05260

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093554.094709

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GCATTTCCAC	GTCAACAGAA	TTGTTTATTG	TGACAGTTAT	ATCTGTTGTC	CCTTTGACCT	4260
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TTGCAGTATG	CTCCACGTGG	TAAGATCCTC	CAAGCTGCTT	TAGAAGTAAC	AATGAAGAAC	4800
GTGGACGTTT	TTAATATAAA	GCCTGTTTTG	TCTTTTGTTG	TTGTTCAAAC	GGGATTCACA	4860
GAGTATTTGA	AAAATGTATA	TATATTAAGA	GGTCACGGGG	GCTAATTGCT	AGCTGGCTGC	4920
CTTTTGCTGT	GGGGTTTTGT	TACCTGGTTT	TAATAACAGT	AAATGTGCCC	AGCCTCTTGG	4980
CCCCAGAACT	GTACAGTATT	GTGGCTGCAC	TTGCTCTAAG	AGTAGTTGAT	GTTGCATTTT	5040
CCTTATTGTT	AAAAACATGT	TAGAAGCAAT	GAATGTATAT	AAAAGC		5086

003354103430

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 717 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

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(ix) FEATURE:
      (A) NAME/KEY: CDS
      (B) LOCATION: 1..717
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ATG Met 1	GCG Ala	CAC His	GCT Ala	GGG Gly 5	AGA Arg	ACG Thr	GGG Gly	TAC Tyr	GAC Asp 10	AAC Asn	CGG Arg	GAG Glu	ATA Ile	GTG Val 15	ATG Met	48
AAG Lys	TAC Tyr	ATC Ile	CAT His 20	TAT Tyr	AAG Lys	CTG Leu	TCG Ser	CAG Gln 25	AGG Arg	GGC Gly	TAC Tyr	GAG Glu	TGG Trp 30	GAT Asp	GCG Ala	96
GGA Gly	GAT Asp 35	GTG Val 35	GGC Gly	GCC Ala	GCG Ala	CCC Pro	CCG Pro 40	GGG Gly	GCC Ala	GCC Ala	CCC Pro	GCA Ala 45	CCG Pro	GGC Gly	ATC Ile	144
TTC Phe 50	TCC Ser	TCC Ser	CAG Gln	CCC Pro	GGG Gly	CAC His 55	ACG Thr	CCC Pro	CAT His	CCA Pro	GCC Ala 60	GCA Ala	TCC Ser	CGC Arg	GAC Asp	192
CCG Pro 65	GTC Val	GCC Ala	AGG Arg	ACC Thr	TCG Ser 70	CCG Pro	CTG Leu	CAG Gln	ACC Thr	CCG Pro 75	GCT Ala	GCC Ala	CCC Pro	GGC Gly	GCC Ala 80	240
GCC Ala	GCG Ala	GGG Gly	CCT Pro	GCG Ala 85	CTC Leu	AGC Ser	CCG Pro	GTG Val	CCA Pro 90	CCT Pro	GTG Val	GTC Val	CAC His	CTG Leu 95	GCC Ala	288
CTC Leu	CGC Arg	CAA Gln 100	GCC Ala	GGC Gly	GAC Asp	GAC Asp	TTC Phe	TCC Ser 105	CGC Arg	CGC Arg	TAC Tyr	CGC Arg	GGC Gly 110	GAC Asp	TTC Phe	336
GCC Ala	GAG Glu	ATG Met 115	TCC Ser	AGC Ser	CAG Gln	CTG Leu	CAC His 120	CTG Leu	ACG Thr	CCC Pro	TTC Phe	ACC Thr 125	GCG Ala	CGG Arg	GGA Gly	384
CGC Arg	TTT Phe 130	GCC Ala	ACG Thr	GTG Val	GTG Val	GAG Glu 135	GAG Glu	CTC Leu	TTC Phe	AGG Arg 140	GAC Asp	GGG Gly	GTG Val	AAC Asn	TGG Trp	432

GGG Gly 145	AGG Arg	ATT Ile	GTG Val	GCC Ala	TTC Phe	TTT Phe	GAG Glu	TTC Phe	GGT Gly	GGG Gly 155	GTC Val	ATG Met	TGT Cys	GTG Val	GAG Glu 160	480
AGC Ser	GTC Val	AAC Asn	CGG Arg	GAG Glu 165	ATG Met	TCG Ser	CCC Pro	CTG Leu	GTG Val 170	GAC Asp	AAC Asn	ATC Ile	GCC Ala	CTG Leu 175	TGG Trp	528
ATG Met	ACT Thr	GAG Glu	TAC Tyr 180	CTG Leu	AAC Asn	CGG Arg	CAC His	CTG Leu 185	CAC His	ACC Thr	TGG Trp	ATC Ile	CAG Gln 190	GAT Asp	AAC Asn	576
GGA Gly	GGC Gly	TGG Trp 195	GAT Asp	GCC Ala	TTT Phe	GTG Val	GAA Glu 200	CTG Leu	TAC Tyr	GGC Gly	CCC Pro	AGC Ser 205	ATG Met	CGG Arg	CCT Pro	624
CTG Leu 210	TTT Phe	GAT Asp	TTC Phe	TCC Ser	TGG Trp	CTG Leu 215	TCT Ser	CTG Leu	AAG Lys	ACT Thr	CTG Leu 220	CTC Leu	AGT Ser	TTG Leu	GCC Ala	672
CTG Leu 225	GTG Val	GGA Gly	GCT Ala	TGC Cys	ATC Ile 230	ACC Thr	CTG Leu	GGT Gly	GCC Ala	TAT Tyr 235	CTG Leu	AGC Ser	CAC His	AAG Lys		717

CCZFB04T53E00

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 239 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met 1	Ala	His	Ala	Gly 5	Arg	Thr	Gly	Tyr	Asp 10	Asn	Arg	Glu	Ile	Val 15	Met
Lys	Tyr	Ile	His 20	Tyr	Lys	Leu	Ser	Gln 25	Arg	Gly	Tyr	Glu	Trp 30	Asp	Ala
Gly	Asp	Val 35	Gly	Ala	Ala	Pro	Pro 40	Gly	Ala	Ala	Pro	Ala 45	Pro	Gly	Ile
Phe	Ser 50	Ser	Gln	Pro	Gly	His 55	Thr	Pro	His	Pro	Ala 60	Ala	Ser	Arg	Asp
Pro 65	Val	Ala	Arg	Thr	Ser 70	Pro	Leu	Gln	Thr	Pro 75	Ala	Ala	Pro	Gly	Ala 80
Ala	Ala	Gly	Pro	Ala 85	Leu	Ser	Pro	Val	Pro 90	Pro	Val	Val	His	Leu 95	Ala
Leu	Arg	Gln	Ala 100	Gly	Asp	Asp	Phe	Ser 105	Arg	Arg	Tyr	Arg	Gly 110	Asp	Phe
Ala	Glu	Met 115	Ser	Ser	Gln	Leu	His 120	Leu	Thr	Pro	Phe	Thr 125	Ala	Arg	Gly
Arg	Phe 130	Ala	Thr	Val	Val	Glu 135	Glu	Leu	Phe	Arg	Asp 140	Gly	Val	Asn	Trp
Gly 145	Arg	Ile	Val	Ala	Phe 150	Phe	Glu	Phe	Gly	Gly 155	Val	Met	Cys	Val	Glu 160
Ser	Val	Asn	Arg	Glu 165	Met	Ser	Pro	Leu	Val 170	Asp	Asn	Ile	Ala	Leu 175	Trp
Met	Thr	Glu	Tyr 180	Leu	Asn	Arg	His	Leu 185	His	Thr	Trp	Ile	Gln 190	Asp	Asn
Gly	Gly	Trp 195	Asp	Ala	Phe	Val	Glu 200	Leu	Tyr	Gly	Pro	Ser 205	Met	Arg	Pro
Leu	Phe 210	Asp	Phe	Ser	Trp	Leu 215	Ser	Leu	Lys	Thr 220	Leu	Leu	Ser	Leu	Ala

Leu Val Gly Ala Cys Ile Thr Leu Gly Ala Tyr Leu Ser His Lys
225 230 235

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 615 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..615

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

ATG GCG CAC GCT GGG AGA ACG GGG TAC GAC AAC CGG GAG ATA GTG ATG	48
Met Ala His Ala Gly Arg Thr Gly Tyr Asp Asn Arg Glu Ile Val Met	
1 5 10 15	
AAG TAC ATC CAT TAT AAG CTG TCG CAG AGG GGC TAC GAG TGG GAT GCG	96
Lys Tyr Ile His Tyr Lys Leu Ser Gln Arg Gly Tyr Glu Trp Asp Ala	
20 25 30	
GGA GAT GTG GGC GCC GCG CCC CCG GGG GCC GCC CCC GCA CCG GGC ATC	144
Gly Asp Val Gly Ala Ala Pro Pro Gly Ala Ala Pro Ala Pro Gly Ile	
35 40 45	
TTC TCC TCC CAG CCC GGG CAC ACG CCC CAT CCA GCC GCA TCC CGC GAC	192
Phe Ser Ser Gln Pro Gly His Thr Pro His Pro Ala Ala Ser Arg Asp	
50 55 60	
CCG GTC GCC AGG ACC TCG CCG CTG CAG ACC CCG GCT GCC CCC GGC GCC	240
Pro Val Ala Arg Thr Ser Pro Leu Gln Thr Pro Ala Ala Pro Gly Ala	
65 70 75 80	
GCC GCG GGG CCT GCG CTC AGC CCG GTG CCA CCT GTG GTC CAC CTG GCC	288
Ala Ala Gly Pro Ala Leu Ser Pro Val Pro Pro Val Val His Leu Ala	
85 90 95	

CCCTGGTTTGGGCGG

CTC CGC CAA GCC GGC GAC GAC TTC TCC CGC CGC TAC CGC GGC GAC TTC	336
Leu Arg Gln Ala Gly Asp Asp Phe Ser Arg Arg Tyr Arg Gly Asp Phe	
100 105 110	
GCC GAG ATG TCC AGC CAG CTG CAC CTG ACG CCC TTC ACC GCG CGG GGA	384
Ala Glu Met Ser Ser Gln Leu His Leu Thr Pro Phe Thr Ala Arg Gly	
115 120 125	
CGC TTT GCC ACG GTG GTG GAG GAG CTC TTC AGG GAC GGG GTG AAC TGG	432
Arg Phe Ala Thr Val Val Glu Glu Leu Phe Arg Asp Gly Val Asn Trp	
130 135 140	
GGG AGG ATT GTG GCC TTC TTT GAG TTC GGT GGG GTC ATG TGT GTG GAG	480
Gly Arg Ile Val Ala Phe Phe Glu Phe Gly Gly Val Met Cys Val Glu	
145 150 155 160	
AGC GTC AAC CGG GAG ATG TCG CCC CTG GTG GAC AAC ATC GCC CTG TGG	528
Ser Val Asn Arg Glu Met Ser Pro Leu Val Asp Asn Ile Ala Leu Trp	
165 170 175	
ATG ACT GAG TAC CTG AAC CGG CAC CTG CAC ACC TGG ATC CAG GAT AAC	576
Met Thr Glu Tyr Leu Asn Arg His Leu His Thr Trp Ile Gln Asp Asn	
180 185 190	
GGA GGC TGG GTA GGT GCA TCT GGT GAT GTG AGT CTG GGC	615
Gly Gly Trp Val Gly Ala Ser Gly Asp Val Ser Leu Gly	
195 200 205	

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 205 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

Met	Ala	His	Ala	Gly	Arg	Thr	Gly	Tyr	Asp	Asn	Arg	Glu	Ile	Val	Met
1				5					10					15	
Lys	Tyr	Ile	His	Tyr	Lys	Leu	Ser	Gln	Arg	Gly	Tyr	Glu	Trp	Asp	Ala
		20						25					30		
Gly	Asp	Val	Gly	Ala	Ala	Pro	Pro	Gly	Ala	Ala	Pro	Ala	Pro	Gly	Ile
		35				40					45				
Phe	Ser	Ser	Gln	Pro	Gly	His	Thr	Pro	His	Pro	Ala	Ala	Ser	Arg	Asp
	50					55					60				
Pro	Val	Ala	Arg	Thr	Ser	Pro	Leu	Gln	Thr	Pro	Ala	Ala	Pro	Gly	Ala
65					70					75				80	

Ala	Ala	Gly	Pro	Ala	Leu	Ser	Pro	Val	Pro	Pro	Val	Val	His	Leu	Ala
				85					90					95	
Leu	Arg	Gln	Ala	Gly	Asp	Asp	Phe	Ser	Arg	Arg	Tyr	Arg	Gly	Asp	Phe
			100					105					110		
Ala	Glu	Met	Ser	Ser	Gln	Leu	His	Leu	Thr	Pro	Phe	Thr	Ala	Arg	Gly
		115					120					125			
Arg	Phe	Ala	Thr	Val	Val	Glu	Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp
	130					135					140				
Gly	Arg	Ile	Val	Ala	Phe	Phe	Glu	Phe	Gly	Gly	Val	Met	Cys	Val	Glu
145					150					155					160
Ser	Val	Asn	Arg	Glu	Met	Ser	Pro	Leu	Val	Asp	Asn	Ile	Ala	Leu	Trp
				165					170					175	
Met	Thr	Glu	Tyr	Leu	Asn	Arg	His	Leu	His	Thr	Trp	Ile	Gln	Asp	Asn
			180					185					190		
Gly	Gly	Trp	Val	Gly	Ala	Ser	Gly	Asp	Val	Ser	Leu	Gly			
		195					200					205			

What is claimed is:

1. A method for increasing the sensitivity of tumor cells to chemotherapeutic agents, comprising the step of introducing an anticode oligomer to tumor cells which express the bcl-2 gene under conditions sufficient to reduce bcl-2 gene expression in said tumor cells.

2. The method of claim 1 wherein said anticode oligomer is an antisense oligonucleotide or an analog thereof.

3. The method of claim 1 wherein the step of introducing comprises:

(a) transfecting said tumor cells with a vector comprising a sequence that encodes said anticode oligomer; and

(b) expressing said anticode oligomer.

4. The method of claim 1 wherein said anticode oligomer has a sequence which binds with a sequence portion of RNA expressed from the bcl-2 gene, which RNA comprises a coding region essentially for bcl-2 protein.

5. The method of claim 4 wherein said sequence portion is a strategic site in pre-mRNA expressed from said bcl-2 gene.

6. The method of claim 5 wherein said anticode oligomer is substantially complementary to and binds to a strategic site in said pre-mRNA.

7. The method of claim 1 wherein said anticode oligomer is brought into contact with said cells under conditions where the concentration of said anticode oligomer is from about 0.001 to about 100 micromolar.

8. A method for killing tumor cells,
comprising the steps of:

- 5 (a) introducing an antisense anticode
oligomer to tumor cells which express the bcl-2 gene
under conditions sufficient to reduce bcl-2 gene
expression in said tumor cells; and
- 10 (b) contacting said tumor cells with an
amount of at least one chemotherapeutic agent sufficient
to kill a portion of said tumor cells, whereby the
portion of tumor cells killed is greater than the portion
which would have been killed by the same amount of said
chemotherapeutic agent in the absence of said
introduction of said anticode oligomer.

15 9. The method of claim 8 wherein said
chemotherapeutic agent is selected from the group of
chemotherapeutic agents consisting of antimetabolites,
alkylating agents, plant alkaloids, and antibiotics.

20 10. The method of claim 8 wherein the step of
introducing comprises:

(a) transfecting said tumor cells with a
vector comprising a sequence that encodes an anticode
oligomer; and

(b) expressing said anticode oligomer.

25 11. The method of claim 8 wherein said
anticode oligomer has a sequence which binds with a
sequence portion of RNA expressed from the bcl-2 gene,
which RNA comprises a coding region essentially for bcl-2
protein.

30 12. The method of claim 11 wherein said
sequence portion is a strategic site in pre-RNA expressed
from said bcl-2 gene.

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13. The method of claim 12 wherein said anticodon oligomer is substantially complementary to and binds to a strategic site in said pre-mRNA.

5 14. The method of claim 8 wherein said anticodon oligomer is brought into contact with said cells under conditions where the concentration of said anticodon oligomer is from about 0.001 to about 100 micromolar.

10 15. A method of inhibiting the growth of cancer cells which express the human bcl-2 gene, comprising the steps of:

15 (a) providing an anticodon oligomer which binds with a sequence portion of RNA expressed from the human bcl-2 gene, which anticodon oligomer when brought in contact with tumor cells expressing the human bcl-2 gene, has the property of reducing the expression of at least one bcl-2 gene product; and

(b) contacting said cells with said anticodon oligomer under conditions sufficient to inhibit growth of said cells.

20 16. The method of claim 15 wherein said anticodon oligomer is an antisense oligonucleotide or analog thereof.

25 17. The method of claim 15 wherein said sequence portion is a strategic site in pre-mRNA expressed from said bcl-2 gene.

18. The method of claim 17 wherein said anticodon oligomer is substantially complementary to and binds to a strategic site in said pre-mRNA.

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19. The method of claim 15 wherein said anticodon oligomer is brought into contact with said cells under conditions where the concentration of said anticodon oligomer is from about 0.001 to about 100 micromolar.

5 20. An anticodon oligomer useful for inhibiting cells expressing the human bcl-2 gene, comprising an anticodon oligomer which binds with a sequence portion of RNA expressed from the human bcl-2 gene, which anticodon oligomer, when brought in contact with tumor cells
10 expressing the human bcl-2 gene, has the property of reducing the expression of at least one bcl-2 gene product and thereby inducing programmed cell death of said tumor cells.

15 21. The anticodon oligomer of claim 20 wherein said anticodon oligomer is an antisense oligonucleotide or an analog thereof.

 22. The anticodon oligomer of claim 20 wherein said sequence portion is a strategic site in pre-mRNA expressed from said bcl-2 gene.

20 23. The anticodon oligomer of claim 22 wherein said anticodon oligomer is substantially complementary to and binds to a strategic site in the pre-mRNA.

25 24. A composition useful for inhibiting cells expressing the human bcl-2 gene, comprising an anticodon oligomer which binds with a sequence portion of RNA expressed from the human bcl-2 gene, which anticodon oligomer, when brought in contact with tumor cells expressing the human bcl-2 gene, has the property of
30 reducing the expression of at least one bcl-2 gene product and thereby inducing programmed cell death of said tumor cells, together with a pharmaceutically acceptable carrier.

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31. The vector of claim 30 wherein said antisense oligonucleotide is substantially complementary to and binds to a strategic site in the pre-mRNA.

ABSTRACT

The present invention provides novel anticode oligomers and methods of using them for controlling the growth of cancer cells expressing the bcl-2 gene.

Figure 1

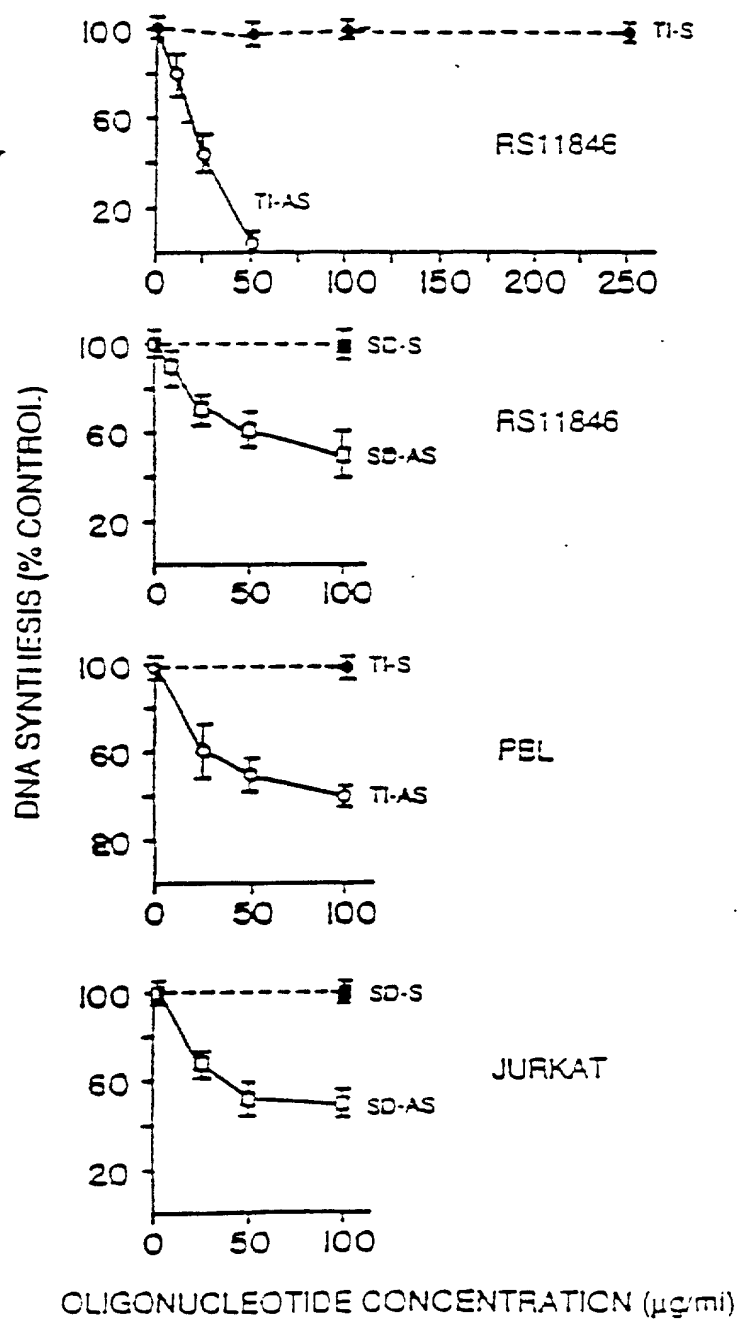
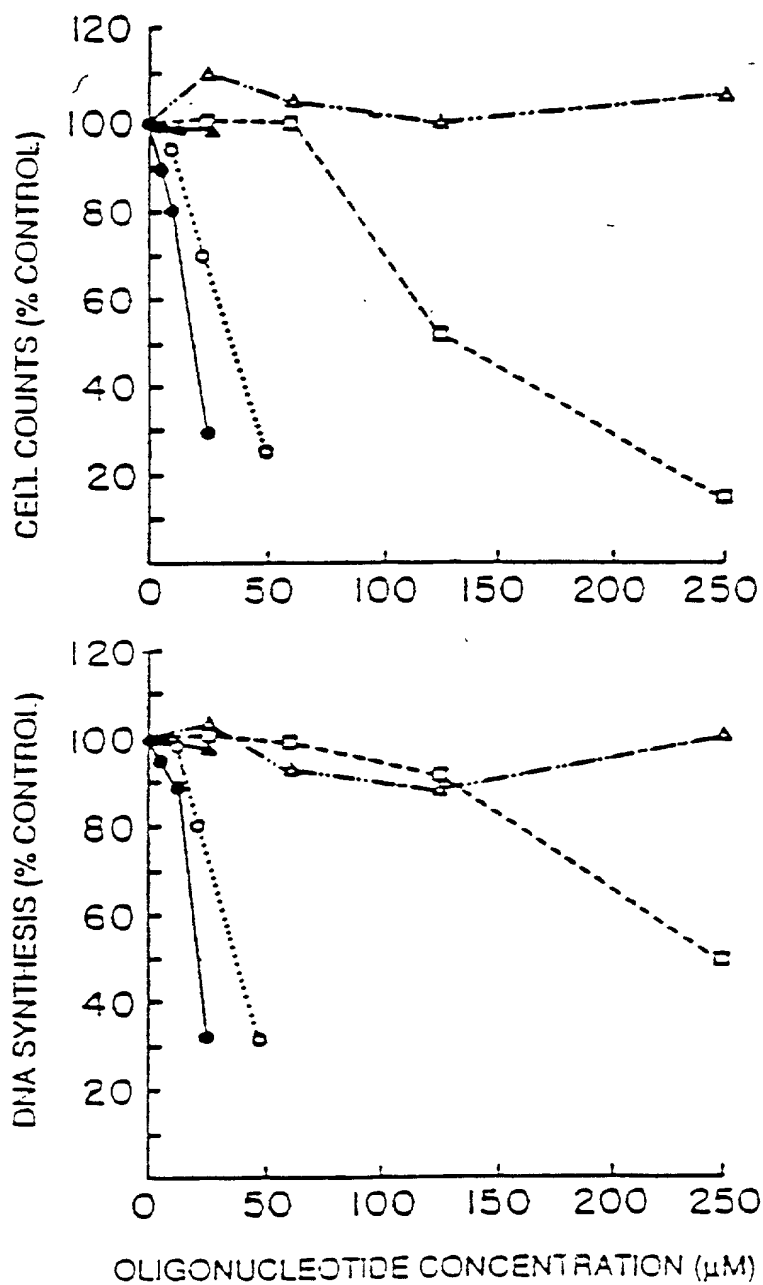
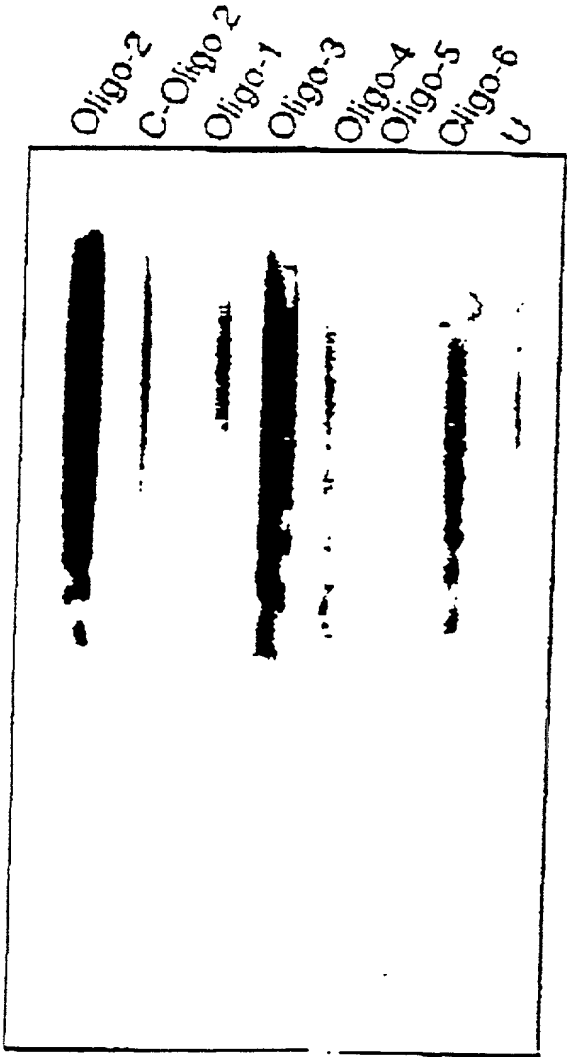


Figure 2



003544 081799 002780 4455/50

FIGURE 3



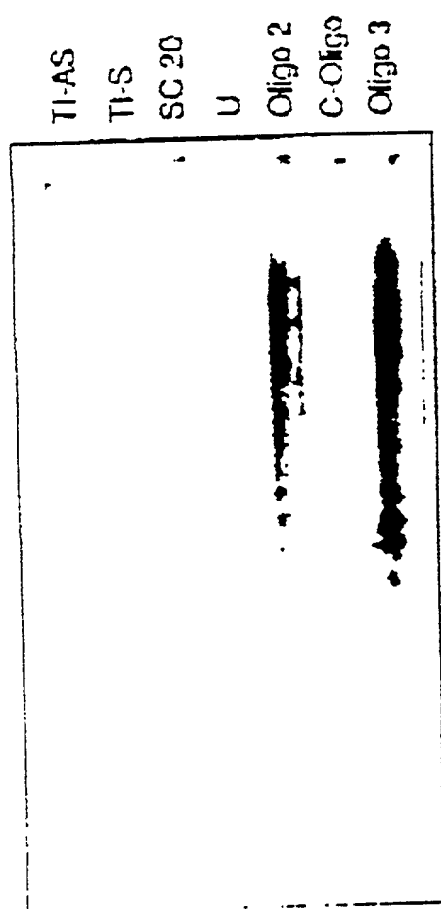


FIGURE 4(a)

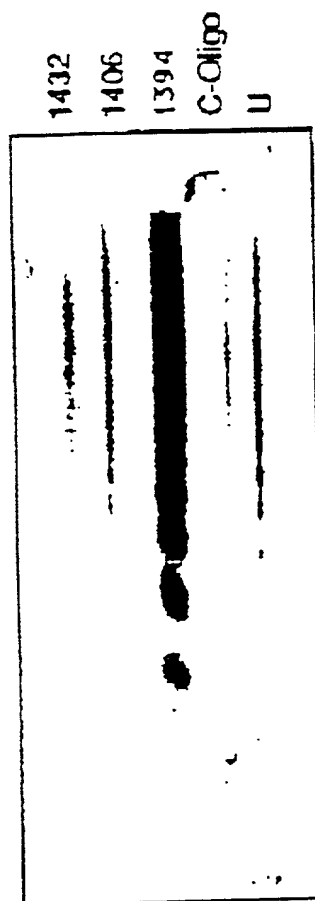
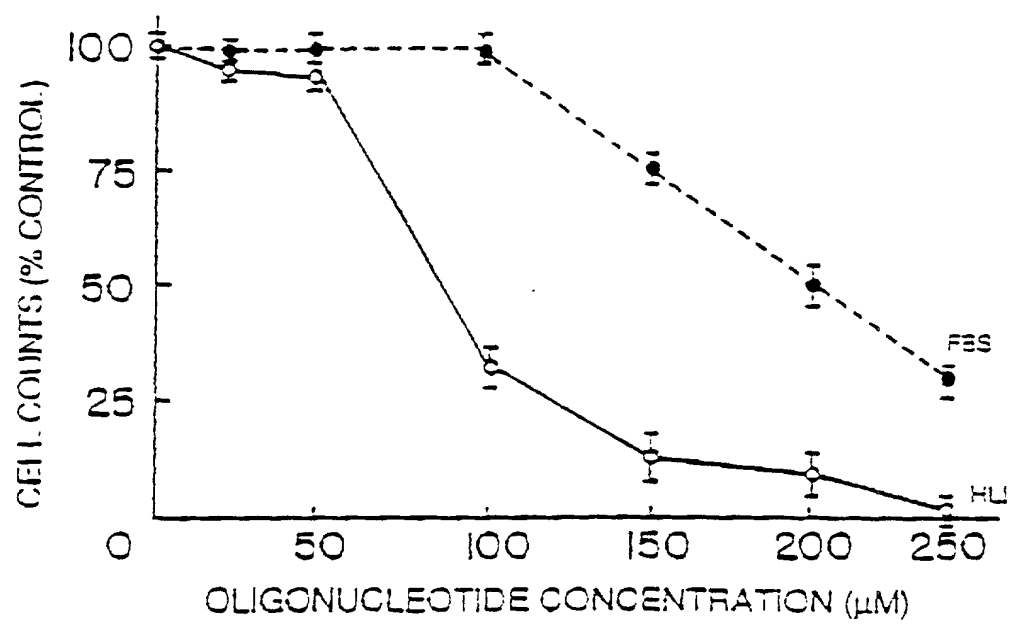


FIGURE 4(b)

FIGURE 5



662150" 4155260

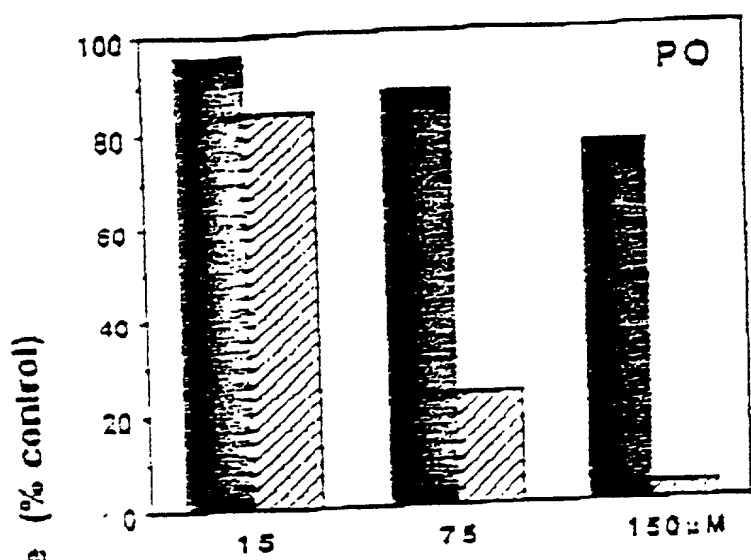


FIGURE 6(a)

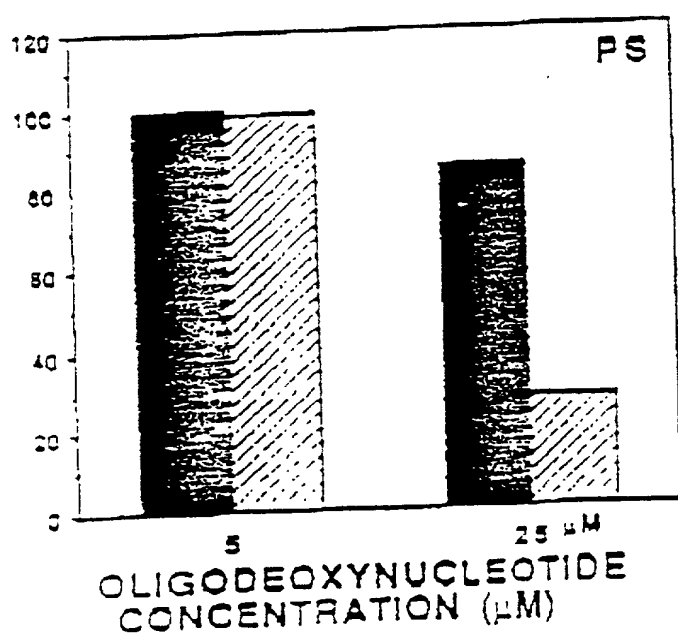


FIGURE 6(b)

FIGURE 7

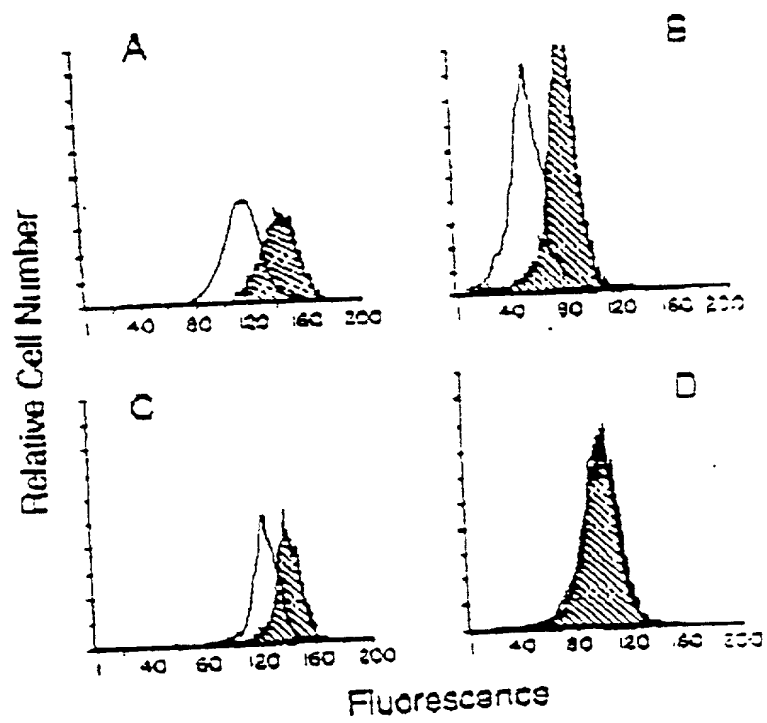


FIGURE 8A

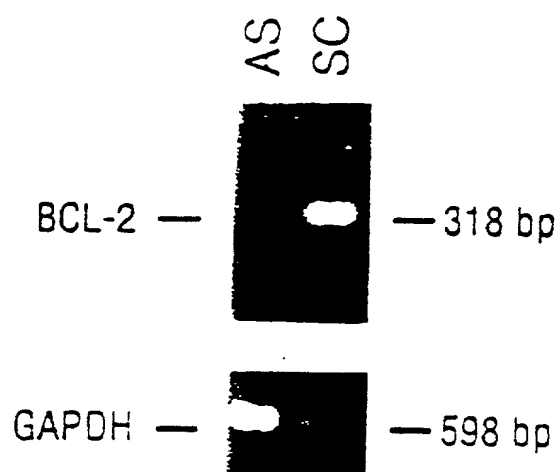


FIGURE 8B

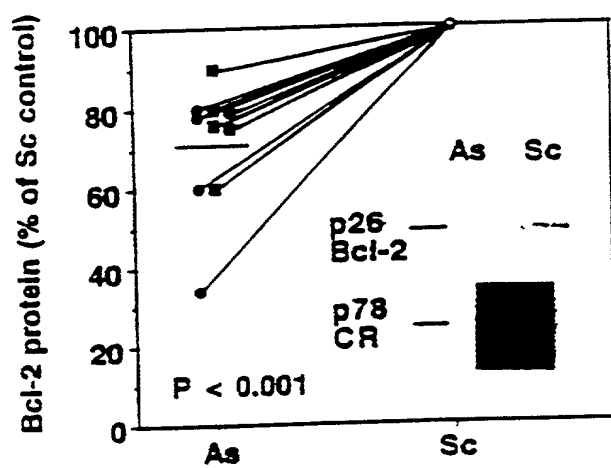


FIGURE 8C

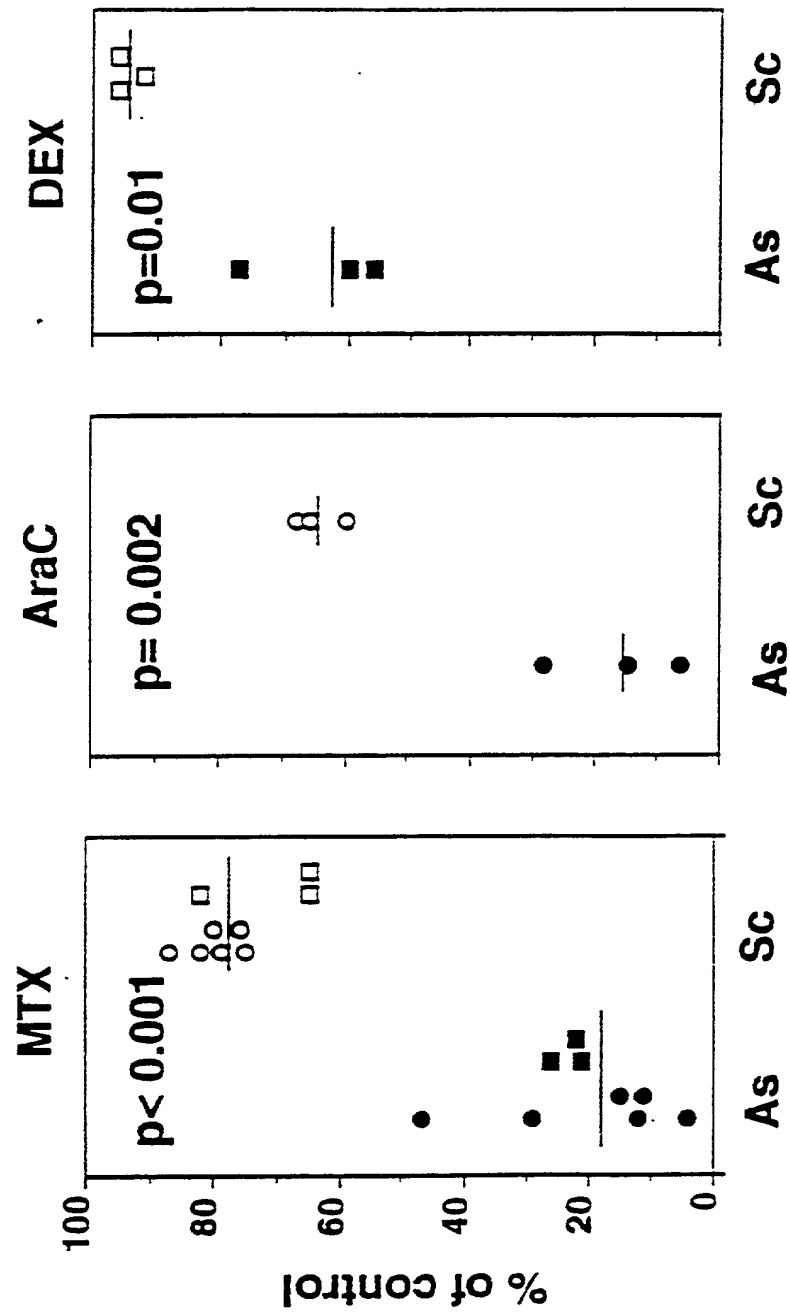


FIGURE 9

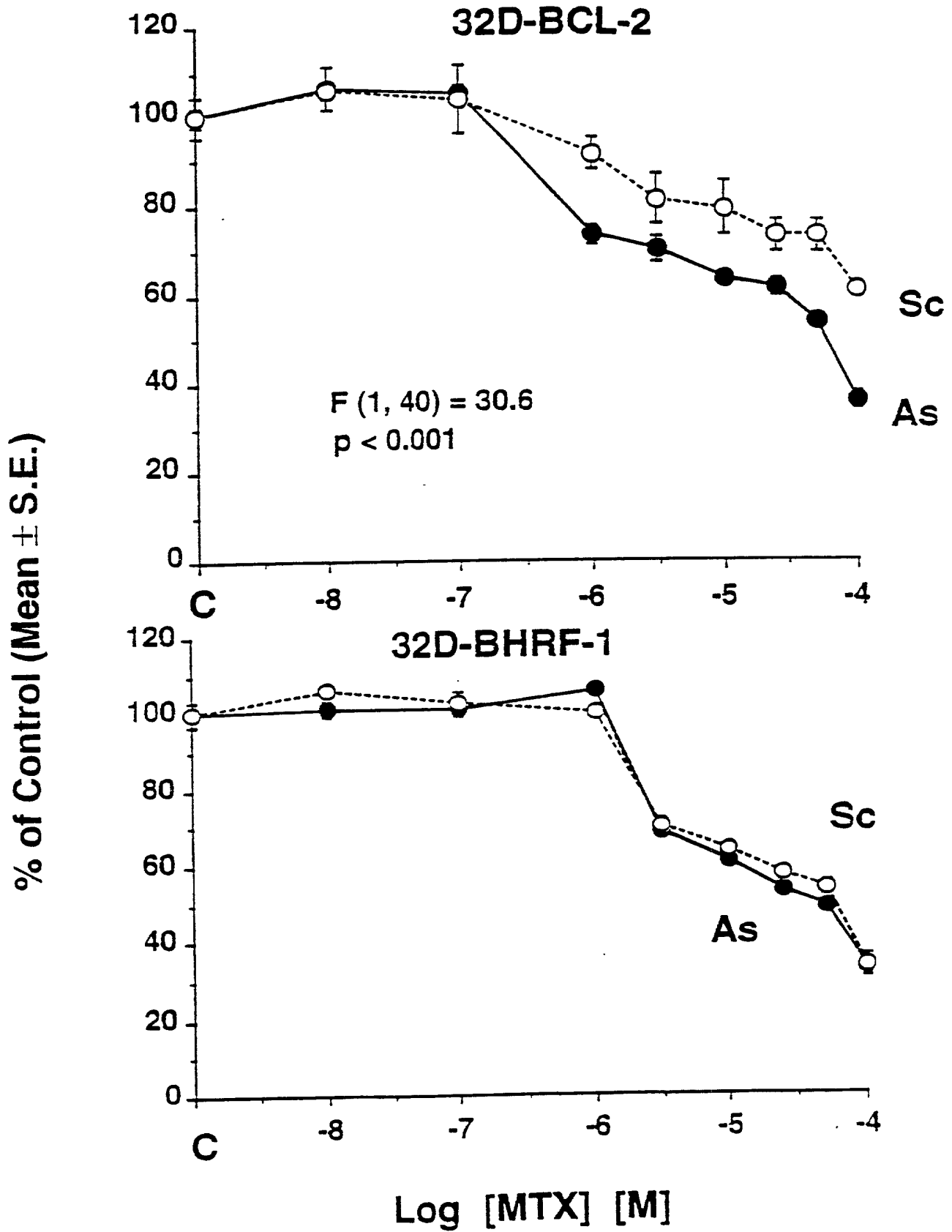
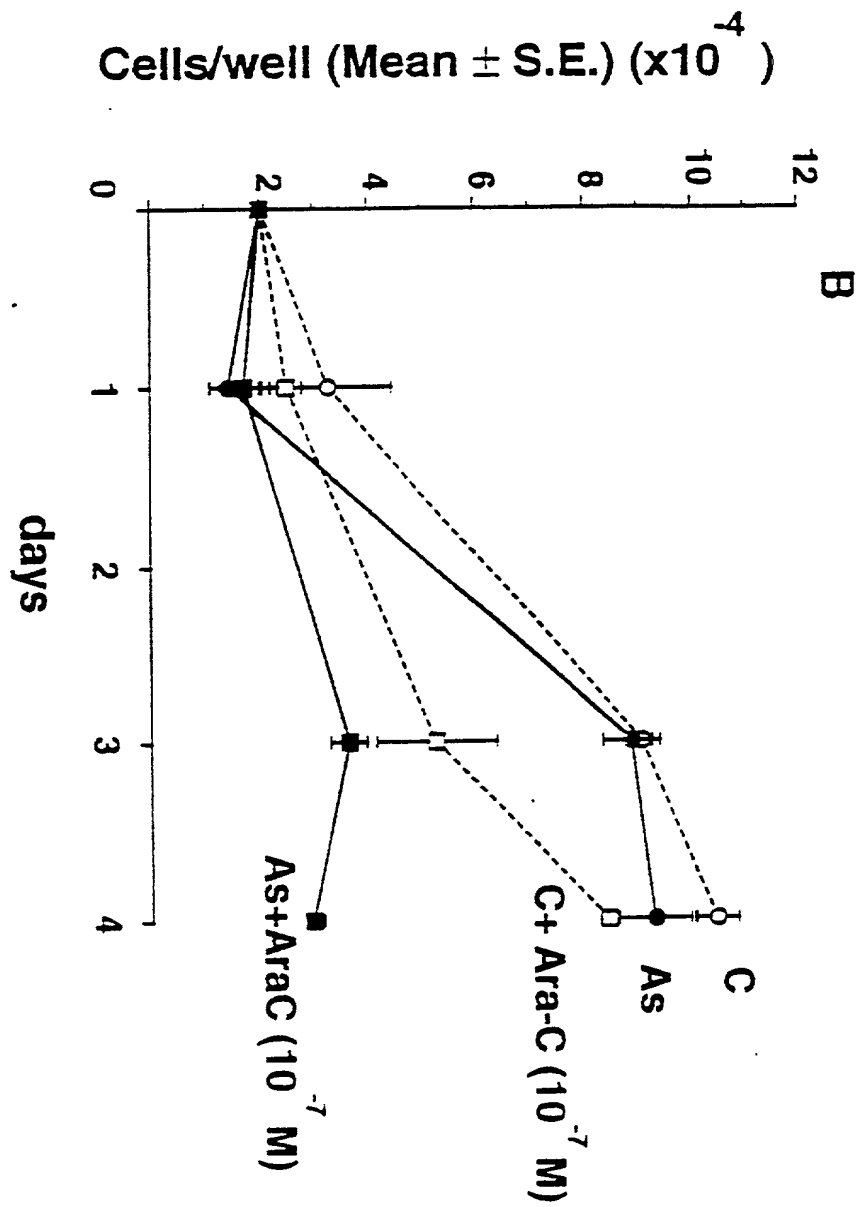


FIGURE 10A



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Cells/well (Mean \pm S.E.) ($\times 10^{-4}$)

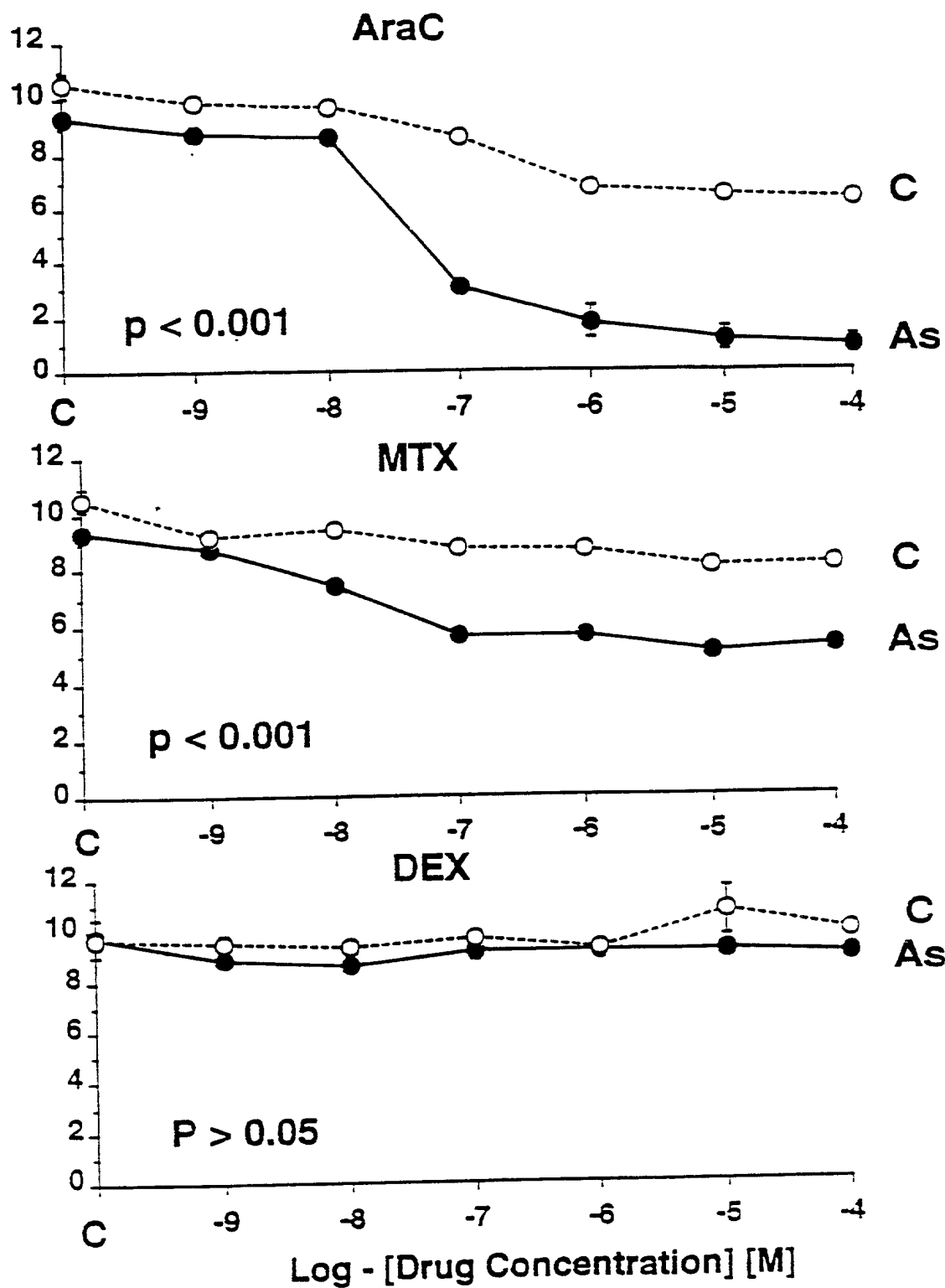


FIGURE 10B

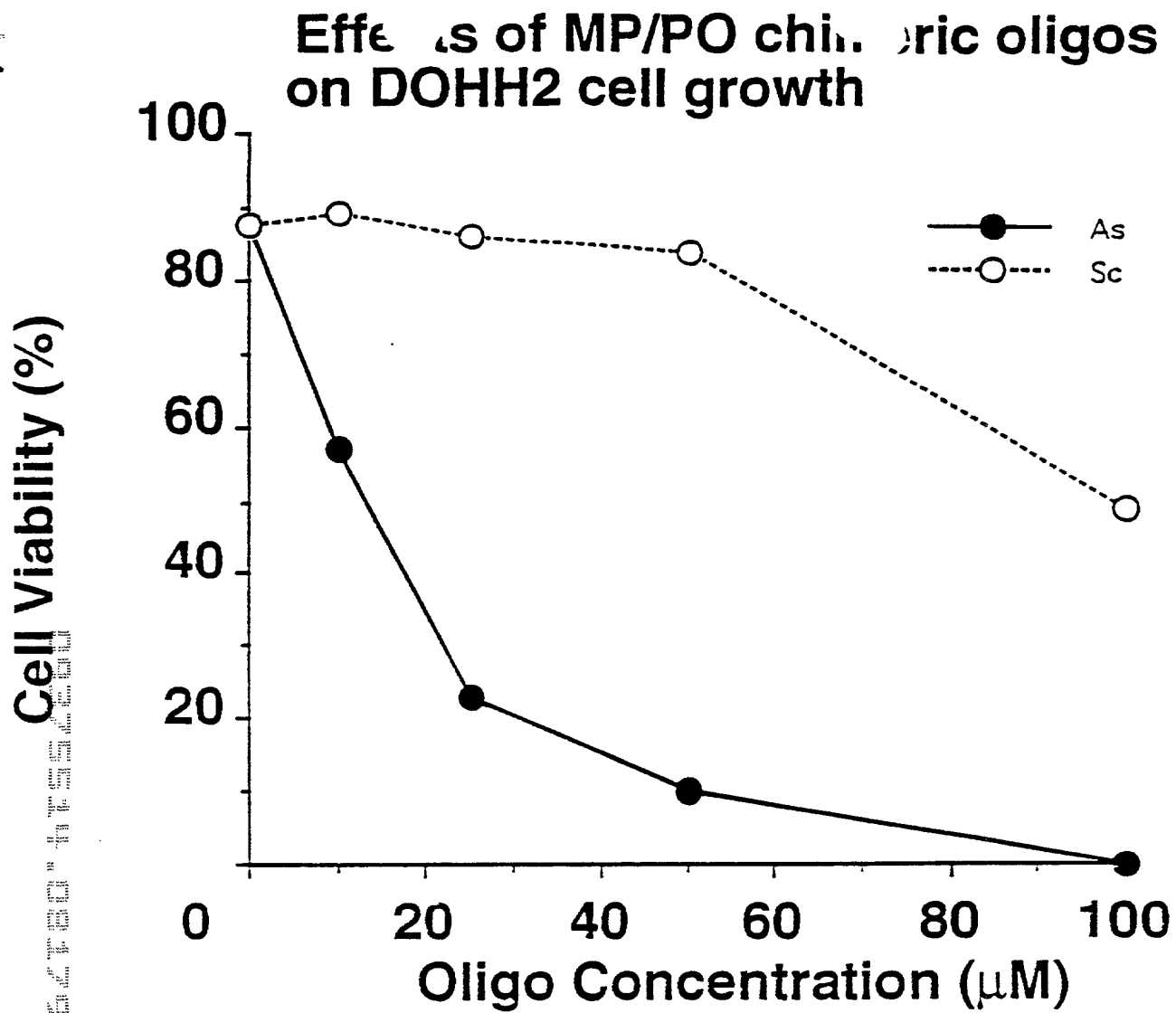


FIGURE 11

**Effects of MP/POchimer. oligos
on MCF7 breast carcinoma cell line.**

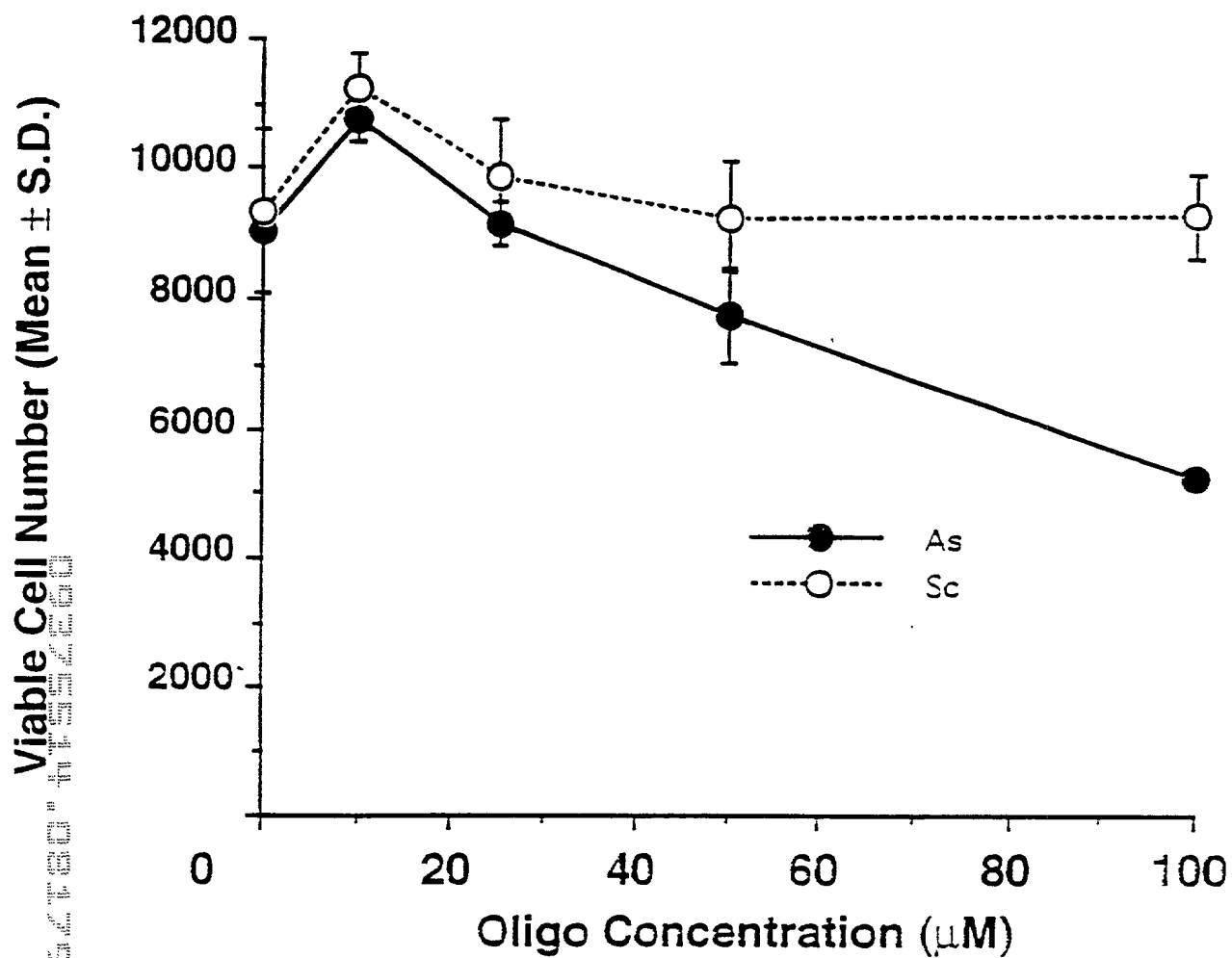


FIGURE 12

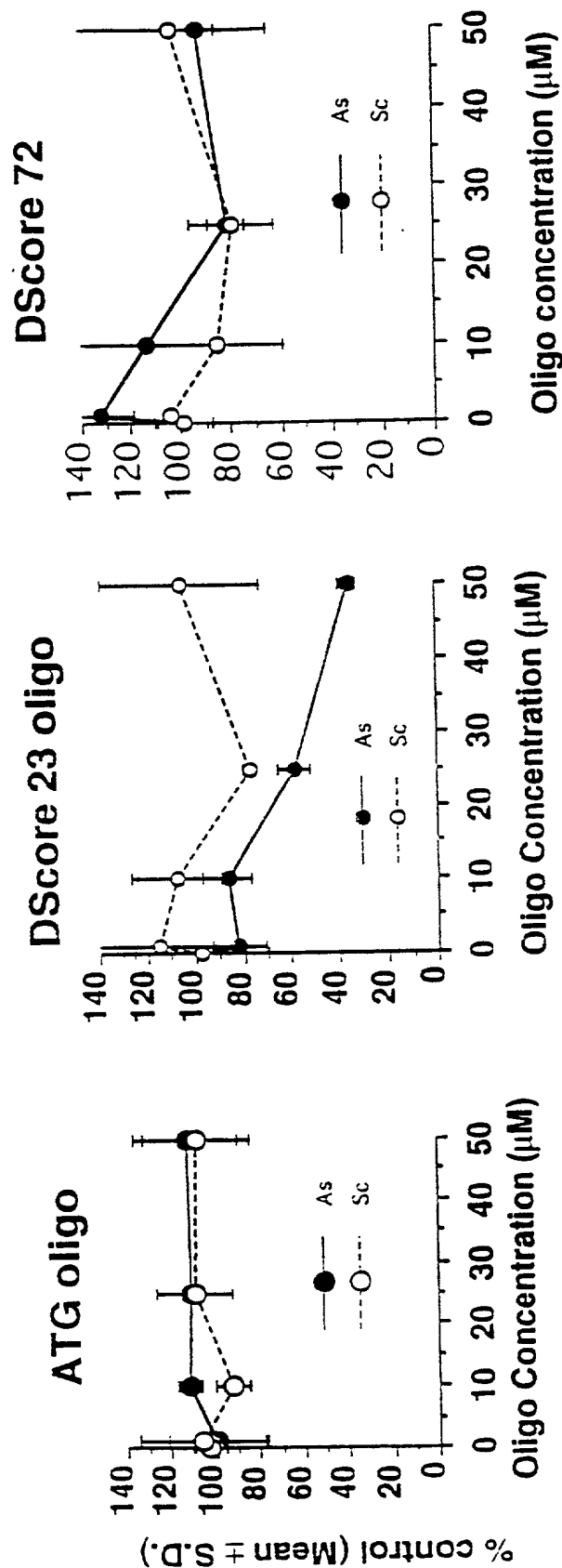


FIGURE 13

As-18' mer
5'-TCTCCAGCGTGGCCAT-3'
Sc-18' mer
5'-TGCACCTACGCTCGGCCT-3'

As23
5'-GCCGGCGGGGGGGGCA-3'
Sc23
5'-GGGGGAGGGGGGGGGG-3'

As72
5'-AGCGGGGGGGGGAGCGC-3'
Sc72
5'-GGGGGGAAGGGGGGGGG-3'

SUBSTITUTE

COPY**Declaration, Power Of Attorney and Petition**

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

REGULATION OF bcl-2 GENE EXPRESSION

the specification of which

☐ is attached hereto.

☒ was filed on SEPTEMBER 20, 1993 as

Application Serial No. 08/124,256

and amended on _____.

☐ was filed as PCT international application

Number _____

on _____,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under Section 119 of Title 35 United States Code, of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Day/Month/Year	Priority Claimed	
_____	_____	_____	<input type="checkbox"/> Yes	<input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes	<input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes	<input type="checkbox"/> No
_____	_____	_____	<input type="checkbox"/> Yes	<input type="checkbox"/> No

We (I) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, We (I) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
<u>07/840,716</u>	<u>FEBRUARY 21, 1992</u>	<u>ABANDONED</u>
<u>07/288,692</u>	<u>DECEMBER 22, 1988</u>	<u>ABANDONED</u>
<u> </u>	<u> </u>	<u> </u>

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Stuart D. Dwork, Registration Number 31,103; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; William B. Walker, Registration Number 22,498; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; and Richard L. Treanor, Registration Number 36,379; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

John C. REED
NAME OF FIRST ~~XXXX~~ INVENTOR


Signature of Inventor

11-2-95
Date

Residence: 4375 Toulomne Place
Carlsbad, California 92008

Citizen of: U.S.A.

Post Office Address: SAME AS ABOVE